THESIS

CONTRACEPTION VACCINATION FOR MARES AND ITS EFFECTS ON CYCLICITY AND ESTROUS BEHAVIOR

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ABSTRACT

CONTRACEPTION VACCINATION FOR MARES AND ITS EFFECTS ON CYCLICITY AND ESTROUS BEHAVIOR

Overpopulation is an issue for wild horses due to limited forage and decreasing water sources. Sterilizing mares without surgical ovariectomy would be cost effective and safer. There are currently no vaccines that cause permanent sterility in mares. Bone Morphogenetic Protein 15 (BMP-15) and Growth Differentiation Factor 9 (GDF-9) are oocyte-specific proteins involved in every stage of follicular development from primordial activation through ovulation. This study investigated the effects of a combination vaccine consisting of these oocyte-specific growth factors, GDF-9 and BMP-15, on mare cyclicity and estrous behavior. We hypothesized that immunization against the combination of these two factors would result in no ovarian cyclicity. Mature, fertile Quarter Horse type mares (n=10/group) each of which had successfully carried a foal within the last 24 months were used. The experiment was conducted from February through September 2018. All mares were vaccinated a total of 5 times starting at week 0, continuing at week 6, 12, 18, and 24. Ten mares received the vaccine consisting of both peptides (GDF-9; SEYFKQFLFPQNEC and BMP-15; QAGSMGSEVLGPSREREGPESNQC) and adjuvant (Seppic Montanide™ Pet Gel A), while control mares received adjuvant alone. All vaccinations were administered IM. Ovarian activity and ovulations were recorded by trans-rectal ultrasonography at least once a week and estrous behaviors were evaluated three days a week by interacting individually with a stallion on a tease rail. Follicle diameters were recorded according to measurements and estrous behavior scored on a 6-point scale (0 = hostile toward stallion – 5 =

actively seeking stallion with associated behaviors). Jugular blood samples were collected prior to each weekly palpation, and serum was aspirated for further investigation of the progesterone levels. All control mares cycled normally with ovulations associated with estrus at approximately 3-week intervals. None of the 10 treated mares ovulated or grew a follicle larger than 20 mm during the 8-month experimental period. Mixed estrous behaviors were noted in a few mares throughout the study. Low progesterone levels in serum samples confirmed these findings and are associated with the presence and/or lack of detected corpra lutea. Future research will focus on the active duration of the vaccination to determine the length of effectiveness. This vaccination could serve as a long-term contraceptive in wild horse herd populations.

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CHAPTER 1 – INTRODUCTION AND BACKGROUND

Introduction

BLM

Wild horses and burros roam federal Bureau of Land Management (BLM) land in the United States. These horses are untamed, untouched and are the true definition of a wild horse. Today, there are thousands of wild horses that roam freely in at least 10 states in the United States (Frey & Thacker, 2018). The bands or herds of horses and burros roam federal BLM lands, forest service land, national parks, and various islands (Frey & Thacker, 2018). The BLM has estimated that there are 88,000 horses and burros on this federal land (Bureau of Land Management, 2019). Wild horses and burros scavenge for forage and water sources and can survive on a wide variety of forage and limited amounts of water (Garrott & Madan, 2013).

In 1971, the government passed a law to protect the wild horses and burros from capture and harassment (Management, 2006). Now these horses and burros are protected by the Act of 1971 and government while being managed by the BLM. There is an estimated 26.9 million acres that the BLM manages (Bureau of Land Management, 2019). The land can support about 26,000 head of wild horses and burros (Smith, 2010).

Issues with Wild Horse Population

Overpopulation has become an increasing issue as herds of wild horses and burros continue to expand on limited land and water source availability. With the lack of public support and costly control methods, the overpopulation issue has continued to escalate. Wild herds are moving onto privately owned land which leads to more problems and the need for capture by the BLM (Frey & Thacker, 2018). The reason these horses are moving to privately-owned lands is due to the lack of natural vegetation on federal BLM lands because of overgrazing by the horses and burros. Natural plants are becoming endangered because horses and burros are eating the plants down to the root and killing the plants. These horses have the ability to survive on various vegetation which is one of the reasons why this population is growing at such a rapid rate (Garrott & Madan, 2013). There are not many natural predators to help manage the population of horses and burros, the only current active predator is the mountain lion (Garrott & Madan, 2013). As a result, a herd can annually grow up to 20% (Ward et al., 2016). Every 4 to 8 years the wild horse population triples if left unmanaged (Garrott & Madan, 2013). Therefore, the natural method to control overpopulation is that herds are running out of forage and water sources and starve to death (Garrott & Madan, 2013). This issue has turned into a large welfare problem that is making this a difficult problem to find a solution for in the equine industry.

A small portion of excess horses get removed and placed in temporary and long term holding pens in various locations throughout the United States, where they are held until adoption events, hosted by the BLM, are put on for the public to purchase these wild horses (Garrott & Madan, 2013). The BLM estimates that there are about 50,000 horses in long term holding pens (Bureau of Land Management, 2019). Although the system in place helps, adoptions unfortunately cannot keep up with the number of horses removed from federal lands (Garrott & Madan, 2013). The need for adoptions is much greater than the number of wild horses that are getting adopted out (Garrott & Madan, 2013). The problem continues to grow because some mares that are captured are pregnant and foal out in temporary holding pens and thus more mouths are then needed to be fed. To maintain these horses in temporary and long-term holding pens is an issue that keeps increasing as more horses and burros are captured. The costs to keep these horses in temporary and long term holding pens is funded by U.S. taxes (Garrott & Madan, 2013). According to BLM, the horses and burros in these pens are costing the U.S. $50 million dollars every year to feed and house (Bureau of Land Management, 2019).

There is a major demand for a greater solution to this wild horse and burro overpopulation problem escalating and spiraling out of control. Euthanasia is an option for these horses as many are in very poor health and welfare conditions, but this is not publicly accepted solution. The need for sterilizing and contracepting the wild horse and burro population is in high demand and is in the process of being researched. The hope is to help slow the growth of these wild horses and burros, so welfare of these animals increases, and they stop starving to death and dying from dehydration. There has been an increase in traffic accidents due to horses and burros scavenging for various forage and water sources, it results in these animals crossing major highways looking for other sources.

Contraceptives

General

Contraceptives have been studied and used in herds of wild horses and burros. Contraceptives have become a way to help control fertility of wild herds (Killian, Thain, Diehl, Rhyan, & Miller, 2008). A vaccination is less invasive and costly compared to surgically removing the ovaries of mares and testicles of stallions. With each of these invasive surgical methods there are numerous consequences that are associated with infections and social behavior changing. More importantly overpopulation will not be affected when castrating stallions due to satellite stallions coving more mares. A satellite stallion is usually a younger stallion that is not part of a herd and stays on the outskirts of herds and breeds mares when the opportunity is there. If alpha stallions are castrated, satellite stallions on the outside of the herds will cover more mares and the same number of foals will be born. Vaccinations can either have a reversible effect on fertility or permanently sterilize mares. There are three options being used today among the various wild herds. These include PZP, PZP-22, and Gonacon – Equine.

PZP

Porcine zona pellucida vaccine (PZP) targets the event of fertilization and prevents the sperm and oocyte from binding (USDA, 2008). PZP is a vaccination against zona protein 3 (ZP3), which is the outermost layer to the oocyte. Having antibodies against ZP3 prevents binding of sperm and oocyte thus disrupting fertilization (Figure 1.1). This vaccination has

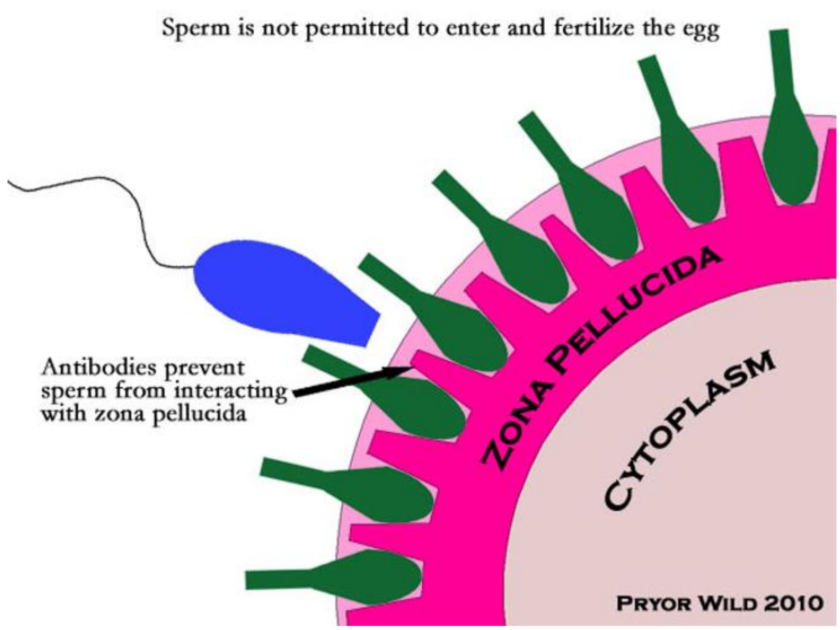

Figure 1.1: PZP Vaccination Diagram
The PZP vaccination disrupts the event of fertilization by ZP3 antibodies not allowing sperm cells to bind with the oocyte (Pryor Wild, 2010).

been used in a variety of species including dogs, coyotes, baboons, burros, wild horses, and white-tailed deer (USDA, 2008). The success of contraception relies on annual boosters (Turner Jr et al., 2008). These boosters require the mares to be vaccinated every year by helicopter or by capturing the entire herd to vaccinate the mares which both methods are costly and cause stress on the herds. The disadvantage of PZP is that the mares continuously display signs of estrus. Mares are pregnant for eleven months out of the year and will display signs of estrus for about 5 days before she is pregnant again and will not show estrus signs. With these vaccinated mares always displaying signs of estrus it is not a normal behavior. Since the event of fertilization is affected mares are always cycling and stallions will always be covering these mares. Continuous signs of estrus is a problem because the mares are continuously being bred. When the mares are always being bred this will increase the odds of a single sperm potentially fertilizing the oocyte if the vaccination is not 100% accurate. This will result in a pregnancy and will not help with the population control that is needed.

PZP-22

Porcine zona pellucida pellet vaccination (PZP-22) has the same concept as PZP vaccine but it is a pellet vaccine as opposed to an injection vaccine (Rutberg, Grams, Turner Jr, & Hopkins, 2017). When mares were primed with the PZP-22 pellet, fertility of that mare (foaling rate) was not any different from the PZP vaccinated mares (Rutberg et al., 2017). The hope of the pellet primer vaccination was to increased longevity of infertility but unfortunately the continuous booster injection vaccination was more effective (Rutberg et al., 2017). Advantages with this PZP-22 is that the concept of not needing annual booster is more cost effective. Disadvantages include that the PZP-22 primer was not more effective than the annual boosters and mares would still need to be caught to administer the pellet and then darted with the PZP as an annual booster. It was named PZP-22 with the goal of the vaccination lasting 22 months but, it is only effective for a year at a time (Rutberg et al., 2017).

GonaCon – Equine

GonaCon vaccine (GnRH vaccine) hinders sex hormone production by building antibodies that bind to gonadotrophin releasing hormone (GnRH) and thus stops the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Baker et al., 2018). GnRH is key in the development and function of the mare's reproduction cycle. GnRH is produced in the hypothalamus and then acts on the pituitary gland to stimulate the production of LH and FSH (Figure 1.2). The vaccination is against GnRH, which results in (FSH) and LH not being produced (Figure 1.2). Once this hypothalamic pituitary gonadal

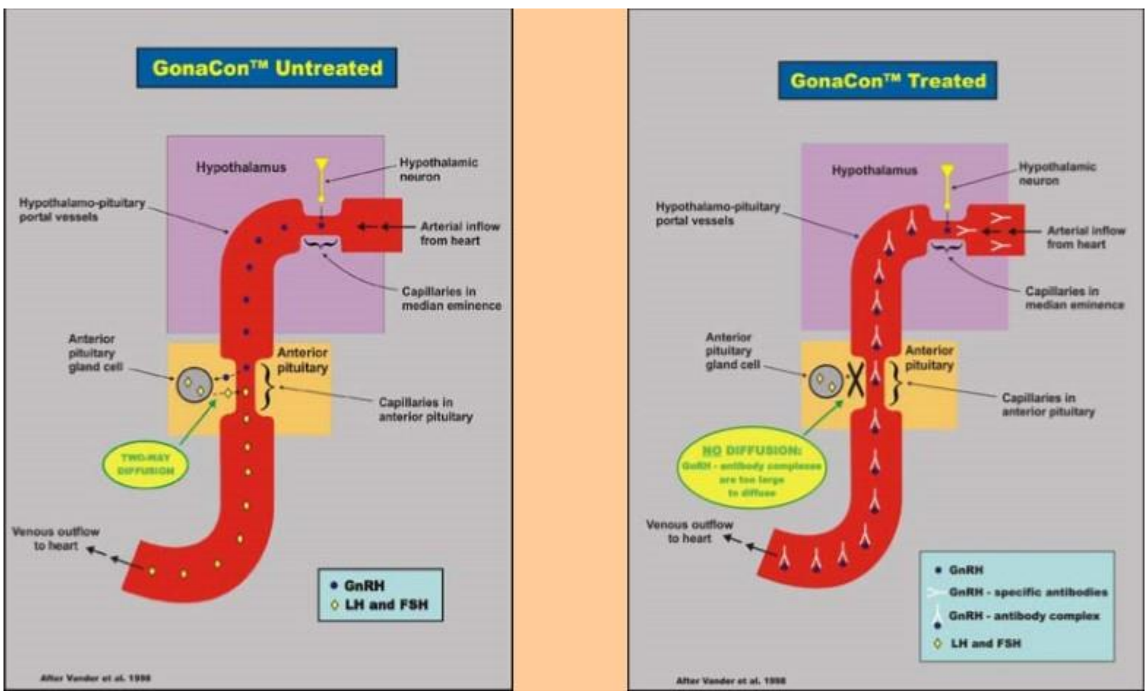

Figure 1.2: GonaCon Vaccination Diagram
The GonaCon vaccination disrupts the production of FSH and LH because GnRH is bound to the antibodies forming complexes that FSH and LH do not recognize (USDA,

axis is disrupted by the GonaCon vaccination, LH and FSH cannot act on the ovaries to produce or ovulate follicles. This vaccination has been successful in squirrels, captive

Norway rats, feral cats and dogs, domestic and feral swine, wild horses, and white-tailed deer (USDA, 2008). In select species including swine and white tailed deer a single shot vaccination lasts up to five years (USDA, 2008). The advantage to this vaccination is that it prevents oocytes from being released and therefore prevents estrus behavior (USDA, 2008). Estrus behavior is displayed with the mare has a dominant pre-ovulatory follicle that is ready to ovulate. Due to loss of the GnRH release from the hypothalamus, sex hormones will not be released, therefore the behaviors associated with those hormones will also be eliminated. The disadvantage includes needing an annual booster for this vaccine to be effective (Baker et al., 2018).

Next Approach

Previous research and studies have targeted various aspects of the reproductive cycle in mares. Targeting the event of fertilization through the PZP vaccination (Figure 1) and targeting the hypothalamic pituitary gonadal axis through the GonaCon vaccine (Figure 2) have been successful but require an annual booster. The next approach that I have researched is targeting follicular development. There are two specific growth factors that are involved in recruiting oocytes into the follicular pool and they bone morphogenetic protein 15 (BMP-15) and growth differentiation factor 9 (GDF-9). By vaccinating against. BMP-15 and GDF-9 this will alter follicular development and potentially stop ovulation. Targeting follicular development might lead to a permanent sterilization vaccination for the wild horses and burros.

Development

Primordial Follicles

Development of oocytes and follicles start before the foal or any mammal is born. Primordial follicles develop once the oogonia become oocytes. The oogonia enter into meiosis after multiple divisions (Pepling, 2006). Once oocytes reach prophase one in meiosis one they are arrested in this stage until puberty (Borum, 1961). As the oocytes are arrested they are individually separated and enclosed in primordial follicles (Pepling, 2006). Each of these primordial follicles consists of an oocyte that is arrested in prophase one of meiosis one and somatic granulosa cells (Pepling, 2006). The formation of these primordial follicles is done by key communication between the pregranulosa cells and the oocytes (Pepling, 2006).

Puberty

Horses reach puberty between one and two years of age. Once the female reaches puberty that mare's brain must receive a signal to start producing gonadotropin releasing hormone (GnRH). GnRH secreting neurons located in the hypothalamus are dormmate until they receive an activating signal (Messager et al., 2005). Kisspeptin is the key signal that activates the GnRH neurons to begin producing GnRH (Messager et al., 2005). As kisspeptin activates the GnRH neurons through the GPR54 receptors this is triggering hypothalamic maturation of the female brain. GnRH is produced in the hypothalamus and travels down to the anterior pituitary to stimulate production of follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH acts on the follicles and stimulates the growth of the follicles and LH aids in the ovulation of the follicle and luteinizes the corpus luteum (CL). Once GnRH is produced and the hypothalamic gonadal axis is active the mare is sexually receptive and can grow and ovulate follicles.

Recruitment – BMP-15 & GDF-9

Once the female is past puberty and kisspeptin has activated the pulsatility of GnRH then the growth and development of follicles occurs. Oocytes arrested in meiosis are recruited into a developing follicular pool. Follicular development includes many different stages that the oocytes and follicles go through (Figure 1.3). During these various stages of development,

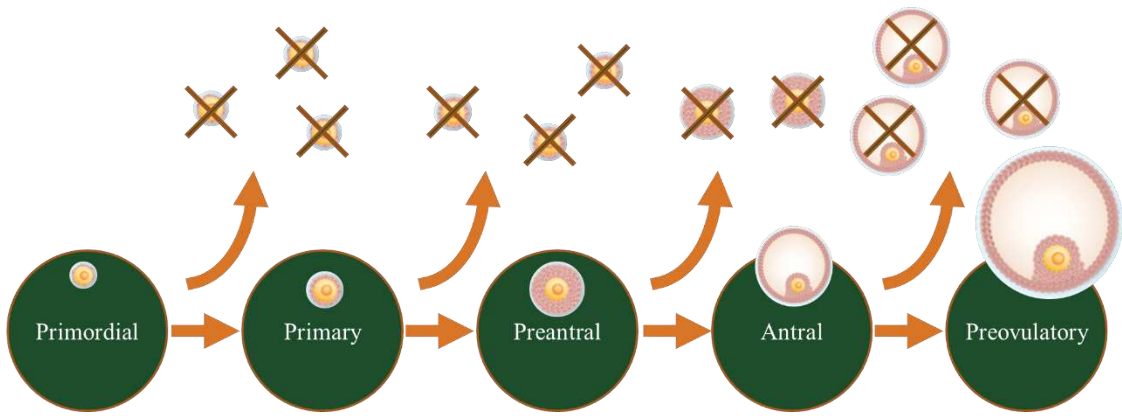

Figure 1.3: Follicular Development
Follicular development from the primordial follicle stage to the preovulatory follicle stage with atresia occurring during the entire development of the select follicle that will ovulate.

the follicles continue to grow and the oocyte within the follicle has more layers of granulosa cells that develop as well as layers of theca cells towards the end of development (Figure 1.3). Atresia, cell death, occurs to follicles that are not healthy and are starved of FSH from the larger more dominant follicles with more FSH receptors. Recruitment occurs through two essential growth factors that are a part of the transforming growth factor-β superfamily (J L Juengel et al., 2004). Growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15), which are essential in fertility and follicular development (J L Juengel et al., 2004). GDF-9 and BMP-15 are oocyte specific proteins and are only secreted by the oocytes (J L Juengel et al., 2004). While these two growth factors are essential in recruitment, development and fertility they are also important in maintaining the follicle as it continues to develop (Figure 1.4). Each of the growth factors has a key role in fertility. GDF-9 and BMP-15 aid with cell growth and differentiation (Kretzschmar, Liu, Jacqueline, & Massagu, 1997). GDF-9 is important in proliferation and differentiation of granulosa cells in primordial follicles and allows for further development (Elvin, Yan, & Matzuk, 2000).

Inhibition of GDF-9 inhibits primordial follicle formation (Wang & Roy, 2004). BMP-15 aids in ovulation rate and when knocked out in mice the females are sub fertile, oocytes get trapped in follicles, females have smaller litter sizes, and there are defects in the embryonic development (Otsuka, McTavish, & Shimasaki, 2011).

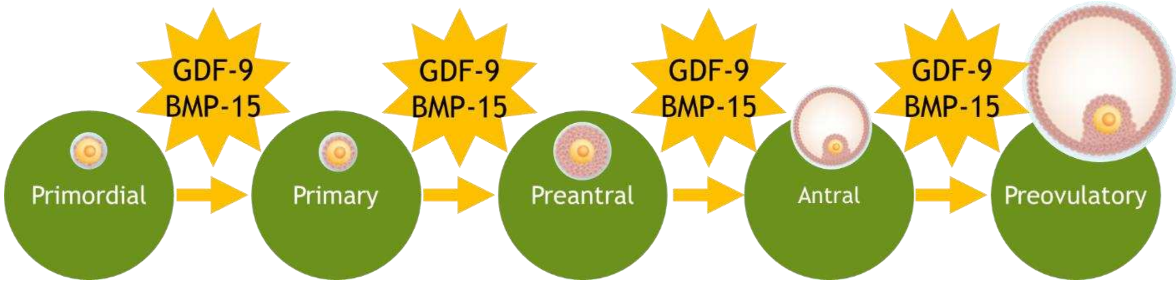

Figure 1.4: Follicular Development with Growth Factors
Follicular development with GDF-9 and BMP-15 growth factors affecting all stages of follicular development and maturation.

Previous studies in various species have targeted GDF-9 and BMP-15 and it effects fertility. In sheep, immunocontraceptive vaccinations against BMP-15 and GDF-9 were administered, and resulted in altered ovulation rates and abnormal follicles in the ewes treated (Jennifer L Juengel et al., 2002). These two growth factors were studied in cattle where they were vaccinated against BMP-15 and GDF-9 had a decrease in the number and size of the antral follicles (Jennifer L Juengel et al., 2009). A study in deer concluded that GDF-9 vaccination did not have any effect year one but in the year two and three, does were infertile (Douglas et al., 2014). In BMP-15 vaccinations there was an increase in fertility and a higher incidence on two or three offspring (Douglas et al., 2014). Although the BMP-15 group had an increase in fertility there was an alteration to the fertility of the does. This could indicate that BMP-15 vaccinations cause more oocytes to be stimulated into the developing follicular pool.

Previous Study – Year 1 and Year 2

Mares were vaccinated with BMP-15 and GDF-9 and monitored for follicular activity and estrus behavior towards stallions (Davis et al., 2018). The year one study concluded in having alterations to the mare's fertility. GDF-9 treated mares there was altered maturation of follicles with smaller preovulatory follicles and more abnormal follicles including hemorrhagic anovulatory follicles and persistent anovulatory follicles (Figure 1.5) (Davis et al., 2018). In mares vaccinated against BMP-15 there was a decrease in the ovulation rate and average follicle size (Figure 1.5) (Davis et al., 2018). The second year that the mare's ovarian

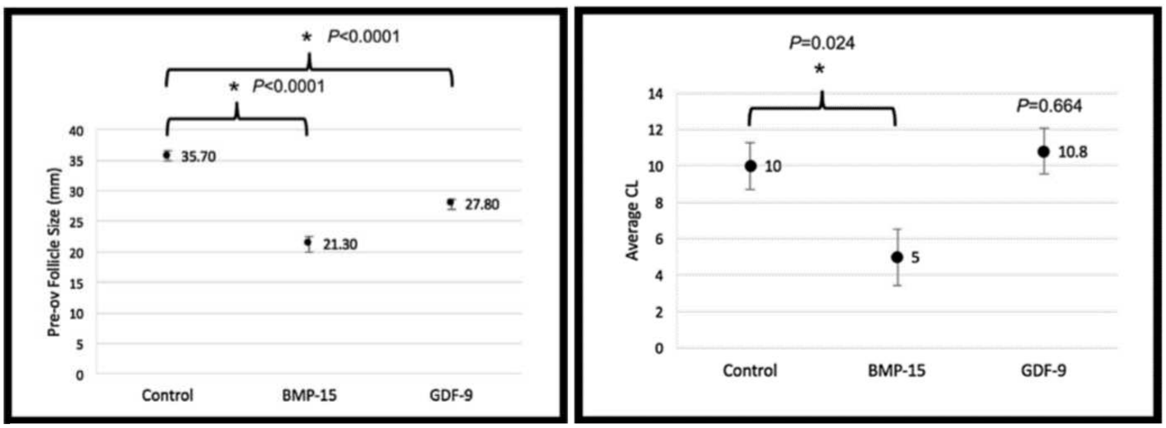

Figure 1.5: Year 1 Largest Follicles Size and Ovulation Rates
Left: Preovulatory follicles were significantly different when compared the control mares (Davis et al., 2018). Right: Ovulation rates in BMP-15 vaccinated mares were significantly different from the control mares (Davis et al., 2018).

activity and estrus behavior was monitored. No additional vaccination or booster vaccinations were administered to the mares. The results on the continuation from year 1 were that the in year 2 the GDF-9 vaccinated mares had a significant decrease in average follicle size and ovulation rate (Figure 1.6). BMP-15 vaccinated mares had a significant decrease in average follicle size and ovulation rate too (Figure 1.6). Comparing results from year 1 to year 2, there is a change in the BMP-15 vaccinated mares and there was a significant decrease in the ovulation rate. This effect is similar to the study in does and the year 1 of the BMP-15 vaccinated does fertility wasn't changed but in years 2 and 3 is where the effect on fertility took place (Douglas et al., 2014). These studies gave a promising effect

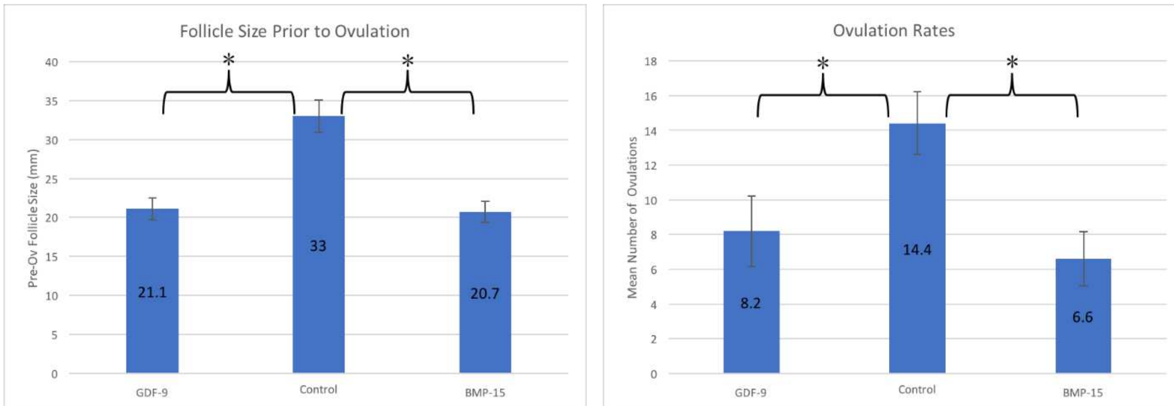

Figure 1.6: Year 2 Largest Follicle Size and Ovulation Rates
Left: Preovulatory sized follicles for the second year of the study were significantly different when compared to the control mares. Right: Ovulation rates for the treatment mares of the second year were significantly different when compared to the control

on mare fertility and the need for further research to determine the combination effect on mares vaccinated with both GDF-9 and BMP-15.

Current Study – Year 3

The study was continued and combined effect of GDF-9 and BMP-15 was done analyzing mares who received the combination vaccination. My hypothesis for this study was that mares that were vaccinated against GDF-9 and BMP-15 would not ovulate. Results were even more significant than the year 1 and 2 studies. Year 3 mares were vaccinated, and ovarian activity and estrus behavior was evaluated using the same protocols as the previous studies. Mares had a significant decrease in the average largest follicle size and had no ovulation. Further explanation of the experimental design and results will be further discussed in chapter 2.

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CHAPTER 2 – JOURNAL ARTICLE[1]

Summary

Overpopulation is an issue for wild horses due to limited forage and decreasing water sources. Sterilizing mares without surgical ovariectomy would be cost effective and safer. There are currently no vaccines that cause permanent sterility in mares. Bone Morphogenetic Protein 15 (BMP-15) and Growth Differentiation Factor 9 (GDF-9) are oocyte-specific proteins involved in every stage of follicular development from primordial activation through ovulation. This study investigated the effects of a combination vaccine consisting of these oocyte-specific growth factors, GDF-9 and BMP-15, on mare cyclicity and estrous behavior. We hypothesized that immunization against the combination of these two factors would result in no ovarian cyclicity. Mature, fertile Quarter Horse type mares (n=10/group) each of which had successfully carried a foal within the last 24 months were used. The experiment was conducted from February through September 2018. All mares were vaccinated a total of 5 times starting at week 0, continuing at week 6, 12, 18, and 24. Ten mares received the vaccine consisting of both peptides (GDF-9; SEYFKQFLFPQNEC and BMP-15; QAGSMGSEVLGPSREREGPESNQC) and adjuvant (Seppic Montanide™ Pet Gel A), while control mares received adjuvant alone. All vaccinations

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were administered IM. Ovarian activity and ovulations were recorded by trans-rectal ultrasonography at least once a week and estrous behaviors were evaluated three days a week by interacting individually with a stallion on a tease rail. Follicle diameters were recorded according to measurements and estrous behavior scored on a 5-point scale (0 = hostile toward stallion – 5 = actively seeking stallion with associated behaviors). Jugular blood samples were collected prior to each weekly palpation, and serum was aspirated for further investigation of the progesterone levels. All control mares cycled normally with ovulations associated with estrus at approximately 3-week intervals. None of the 10 treated mares ovulated or grew a follicle larger than 20 mm during the 8-month experimental period. Mixed estrous behaviors were noted in a few mares throughout the study. Low progesterone levels in serum samples confirmed these findings and are associated with the presence and/or lack of detected corpra lutea. Future research will focus on the active duration of the vaccination to determine the length of effectiveness. This vaccination could serve as a long-term contraceptive in wild horse herd populations.

Introduction

Wild horse overpopulation has become an increasing issue as the herds continue to expand on limited land and water source availability. Since the Wild Free-Roaming Horses and Burros Act in 1971, wild horses are protected (Management, 2006). Horses have few predators and high foaling rates are resulting in a herd annually growing up to 20% (Ward et al., 2016). Every 4 to 8 years the horse population triples if left unmanaged (Garrott & Madan, 2013). The U.S. Bureau of Land Management (BLM) has determined that the capacity for the allotted land that wild horses can roam is 26,000 animals (Smith, 2010). As of 2019, the estimated population of the horses is 88,000 (Bureau of Land Management, 2019). With the lack of public support and costly control methods the overpopulation issue has escalated. Contraceptives are in the process of being researched and administered to wild horses. Porcine zona pellucida (PZP vaccine) and GonaCon (GnRH vaccine) are two current immunocontraceptives for mare contraception. PZP targets the event of fertilization and prevents the sperm and oocyte from binding (USDA, 2008). GonaCon hinders sex hormone production by building antibodies that bind to GnRH thus stops production of FSH and LH (Baker et al., 2018). In this study, we have targeted the ovary and the growth factors that control the oocyte maturation and follicular development resulting in an ovulation and a corpus luteum (CL). Growth Differentiation Factor 9 (GDF-9) and Bone Morphogenetic Protein 15 (BMP-15) are oocyte specific proteins and are essential in fertility and follicular development (J L Juengel et al., 2004). While these two growth factors are essential in recruitment, development and fertility they are also important in maintaining the follicle as it continues to develop. Oocytes secrete GDF-9 and BMP-15 and in sheep that received a vaccination against GDF-9 and BMP-15 and caused sterility (Douglas et al., 2014). Davis et al. concluded that mares that had immunization against BMP-15 had decreased ovulation rate and follicle size, whereas immunization against GDF-9 had altered estrous behavior and follicle maturation and there was an increase in abnormal follicles (Davis et al., 2018). This study investigated the effects of a combination vaccine consisting of oocyte-specific growth factors, BMP-15 and GDF-9, on mare cyclicity and estrous behavior. We are studying the effects of vaccinating against GDF-9 and BMP-15 with both peptides and adjuvant. We hypothesized that immunization against the combination of GDF-9 and BMP-15 would result in no ovarian cyclicity in the mares.

Materials and Methods

Horse Care

All animal procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC# 18-8094A). Mares were obtained from Abraham Equine Inc. in Canadian, Texas. Mares (n = 20) were housed at the Colorado State University Equine Reproduction Laboratory in Fort Collins, CO for the duration of this experiment. Mares were maintained on a dry lot pasture and fed grass-alfalfa mix hay with free choice salt and mineral supplement. Healthy reproductively sound Quarter Horse type mares ranged in age between 12 and 17 each of which had successfully produced a foal within the last 24 months were used. Hooves were trimmed by a farrier every 8 – 10 weeks.

Experimental Design

Treatment mares (n = 10) received a vaccination and four booster vaccinations during the experiment on weeks 0, 6, 12, 18, and 24. The treatment mare observation period ranged from December 11, 2017 through September 14, 2018. Control mares (n = 10) were a part of our previous study (Davis et al., 2018) where three groups (n = 10/group) were randomly assigned to the treatments of control, GDF-9 and BMP-15. Control mare observational period ranged from February 4, 2016 through September 13, 2016 (Davis et al., 2018). Control mares were administered a placebo of phosphate buffered saline with the adjuvant four times at weeks 0, 6, 12, and 18 (Davis et al., 2018).

Immunization Protocol

Treatment mares were vaccinated with the combination of BMP-15 and GDF-9 peptides conjugated to keyhole limpet hemocyanin (KLH) in Seppic Montanide™ Pet Gel A adjuvant, while the horses in the control group received adjuvant and phosphate buffered saline. BMP-15 peptide consisted of a 24 amino acid sequence (QAGSMGSEVLGPSREREGPESNQC) of a mature protein and the GDF-9 peptide was a 14 amino acid sequence (SEYFKQFLFPQNEC) of a mature protein [Celtek Bioscience, Franklin, TN (1st vaccination); Life Technologies Corporation, Carlsbad, CA (2nd, 3rd, 4th, and 5th vaccinations)]. Both sequences are 100% homologous to mature proteins in horses and 80% or 100% homologous to sequences used in studies in sheep (Jennifer L Juengel et al., 2002) and deer (Douglas et al., 2014) for BMP-15 and GDF-9, respectively (Davis et al., 2018). The KLH was used as the carrier protein to improve immunogenicity of peptides. The vaccination formulation contained 1000 µg of peptide-KLH conjugate in a total of 2mL volume per vaccination. The vaccines were administered in the cervical musculature of the neck using a 20-gauge needle. The site of injection was alternated from left to right side at each vaccination for all mares. The injection sites were then closely monitored daily for a week post injection and three times a week after that until the next vaccination. Vaccines were administered on weeks 0, 6, 12, 18, and 24 for the treatment mares starting on December 11, 2017 and for the control mares the vaccines were administered on weeks 0, 6, 12, 18 starting on February 4, 2016 (Davis et al., 2018).

Blood Sample Collection

Blood samples were collected every week for 40 weeks to measure individual antibody responses to the vaccination and circulating progesterone levels. For each sample, 20 mL of jugular blood was drawn from each mare using a 20-gauge 1.5” needle into two 10 mL blood tubes (Medtronic Animal Health; Minneapolis, MN). Samples were incubated at room temperature for at least two hours to allow for separation of sera and red blood cells. The samples were then centrifuged at 2,500g for ten minutes to separate the blood components. Serum was aspirated and pooled from the two collection tubes and transferred into one 15 mL conical tube for each mare. The serum was then centrifuged at 5,250g for thirty minutes to further eliminate any debris. 1 mL samples were then aliquoted into four different 1.7 mL eppendorf tubes and stored at -80°C until further processing.

Antibody Responses

Control mares received their vaccinations on February 4, 2016, March 17, 2016, April 28, 2016, and June 9, 2016. Treatment mares received their vaccinations on December 11, 2017, January 22, 2018, March 7, 2018, April 18, 2018 and May 30, 2018. Injection reaction sites were observed daily for a week post vaccination. Measurements of the injection site swelling was recorded and closely monitored. None of the mares abscessed or became ill post vaccination. All the injection site swelling bumps regressed within 2 weeks following the vaccination. The combined vaccination of BMP-15 and GDF-9 the antibody assay that was performed on these samples were ran on the BMP-15, GDF-9, and controls. For BMP-15 the plates were coated with 10 ng/mL of BMP-15 peptide and run on a VarioSkan Flash plate reader and the serum samples final dilution factor was 1:10,000. For GDF-9, the plates were coated with 5 ng/mL of GDF-9 peptide and run on a VarioSkan Flash plate reader and the serum samples final dilution factor was 1:5,000. For controls, the positive and negative pools were made and ran in 2017 under the same protocol. To account for the plate background the mean, standard deviation, and coefficient of variation from corrected data and subtracted the assay PBS mean for each BMP-15 and GDF-9 assays on all the plates to get the corrected optical density. Outliers were checked for within the plate and within the PBS wells and within the positive and negative controls, GDF-9 had one outlier and there weren't any re-runs of any samples.

Estrous Cycle Behaviors

Estrous behaviors were observed and recorded three times a week during the regular breeding season (February – September 2018). All mares were placed head to tail in a tease rail set up at the Colorado State University Equine Reproductive Laboratory in Fort Collins, Colorado. One sexually excited stallion was used to tease the mares on an individual basis. Four different stallions were used over the course of the study on a rotational schedule. The stallion was presented to the mares starting at their head and spent individual time with each mare. A consistent tease scoring system was used for the duration of the experiment. Mare responses to the stallion were scored on a 0 – 5-point scale. These tease scores were used to compare the days "in estrus" over the course of the experiment. Sexual receptivity was based on the scale and how they interacted with the stallion (Table 2.1). When mares were scored with a 3, 4, or 5 then she was brought into the palpation stocks and evaluated for ovarian activity.

Status	**Tease Score**	**Behavior Description**
Not in estrus	0	Pinned ears, a swishing tail, bared or grinding teeth, kicking, or general discontent with stallions' presence
Not in estrus	1	Indifferent towards stallion – not showing interest or anger of his presence
Not in estrus	2	Somewhat interested behavior with a raised tail but with no urination or active teasing behavior
In estrus	3	Delayed teasing behavior and interest in the stallion, with raised tail, urination, or squatting posture only <u>after</u> interaction with stallion has ended

In estrus	4	Estrus behavior during interaction with the stallion including urination, squatting, winking of clitoris, posturing and raising tail
In estrus	5	Intense teasing behavior beginning prior to the interaction with the stallion with urination, squatting, and raising tail

Table 2.1: Tease Scores
Description of the tease scoring system based on the mare sexual behavior towards the stallion. The mare's interaction with the stallion was evaluated and recorded based on the 0 – 5-point scale. The numerical tease scores are correlated with the "not in estrus" or "in estrus" at each observation and interaction with the stallion.

Ovarian Activity and Records

Ovarian activity of mares was observed and recorded on at least a weekly basis for 28 weeks, unless the mare teased to a stallion (with a score of 3, 4, or 5) then she was evaluated again on the day of estrus behavior. Trans-rectal ultrasonography was conducted with an Aloka 500V equipped with a 7.5 mhz linear transducer. Number and size of ovarian structures including corpora lutea and follicles were identified, noted and recorded into WiseOption computer software at each evaluation. Abnormal follicles and ovarian activities were also recorded over the course of the study. Abnormal follicles included persistent anovulatory follicles, hemorrhagic anovulatory follicles, and follicles with fibrin strands. Abnormal follicles that do not mature or develop properly will result in an unhealthy oocyte. Uterine edema was recorded using a 4-point scale with 0 representing no folds and 4 representing very large endometrial folds. Uterine fluid was described and recorded by both a quantity and echogenic scale. The quality of the fluid was scored using trace, small, medium or large scale and the echogenicity was scored on a 0 – 4 scale with 0 representing clear fluid with minimal echogenicity and 4 representing very cloudy fluid with high amounts of echogenicity. Follicles will grow and mature before ovulating between 40 – 60 mm in diameter. Images show multiple small sized follicles in a treatment mare and a control mare at two different times during the experiment (Figure 2.1).

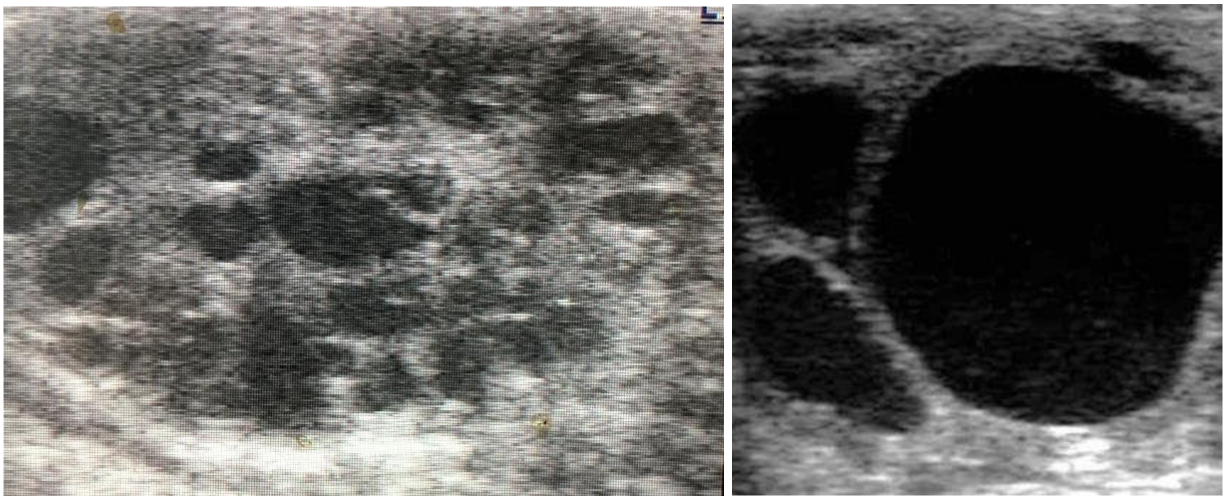

Figure 2.1: Abnormal and Normal Mare Ovary Images
Two still images of an ovary of one treatment mare and one control mare and pictured are numerous small follicles ranging from a 3 – 8 mm in diameter (left) and large follicles ranging from 35 to 15 from a control mare (right).

Progesterone Analysis

The 1mL aliquoted serum samples from the blood collections were stored at -80$^{\circ C}$ until progesterone levels were analyzed. The samples were sent to New Mexico State University in Las Cruces, NM for analysis. Serum progesterone concentrations was analyzed by solid-phase RIA using components of commercial kits manufactured by MP Biomedical (Solon, OH, USA) as described by Castanon et al. (2012) with modifications for horse serum. Briefly, 100 µL of serum was used to determine the P_4 concentration. All tubes were normalized with 0.5 mL assay buffer and each sample was assayed in duplicate. Each tube received 1.0 mL of tracer, vortexed and incubated at room temperature for 24 hours. Tubes were decanted and counted for one minute in a Packard Cobra II gamma counter.

Statistical Analysis

The comparison of tease scores of 3, 4, and 5 were significant with a p-value < 0.0001 and was compared using a two-sample t-test in the JMP Pro 13. Comparisons of the largest follicle sizes between control and treatment mare were significant and was compared using two-sample t-tests in the JMP Pro 13 with p-value < 0.0001. The average progesterone levels between the treatment groups is significant with a p-value < 0.0001 and was compared using a 2-sample t-test in JMP Pro 13.

Results

Antibody Response

A classic positive antibody response was produced by the mares vaccinated against BMP-15 and GDF-9. The mares responded with an increase in their antibody titer following each administration (Figure 2.2). Local inflammation of the injection sites was common among mares however the swelling subsided in within a week of administration. There was no incidence of the vaccination site abscessing post injection.

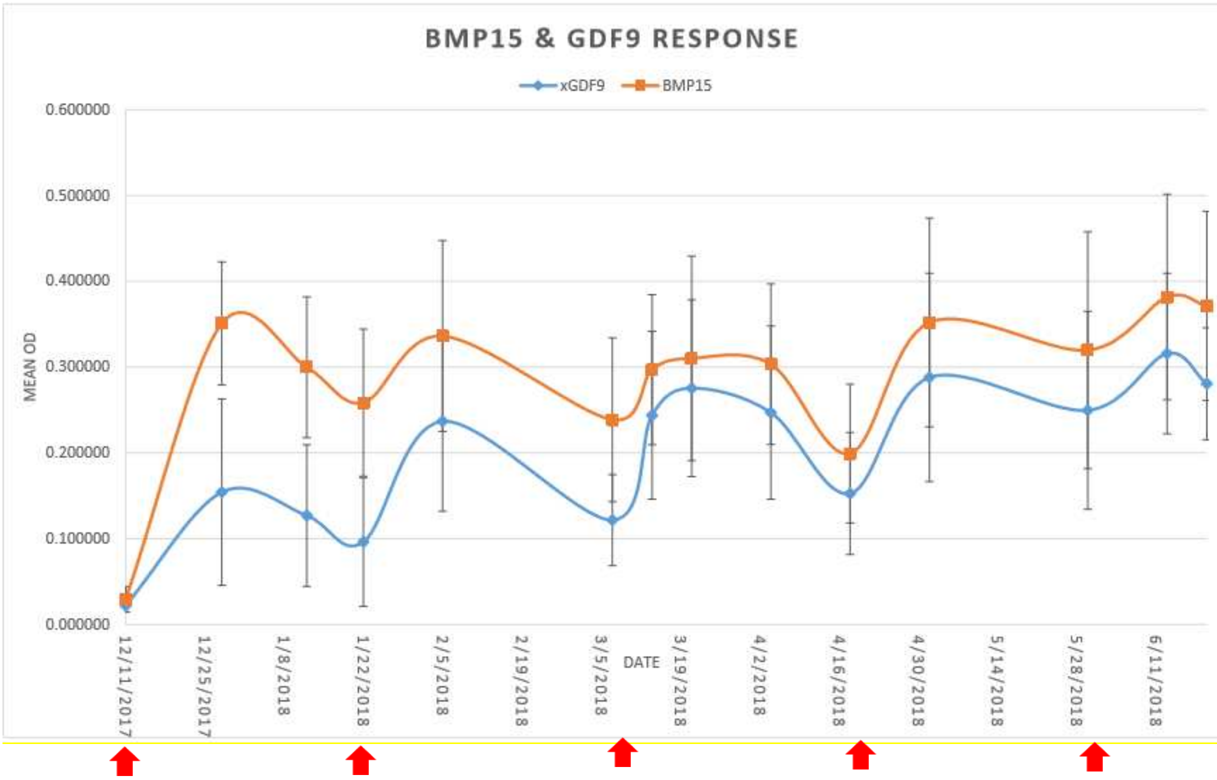

Figure 2.2: Antibody Responses
Mares were vaccinated on December 11, 2017, January 22, 2018, March 7, 2018, April 18, 2018 and May 30, 2018 (indicated by the arrows). Following each vaccine, the mares had a classic antibody response where titer levels increased with each vaccination.

Estrous Behavior

Fewer mares demonstrated in estrus teasing behavior overall on the course of the experiment n=10/group (Figure 2.3). Control mares displayed all categories of the tease scoring system (blue bars, Figure 2.3) which indicated that those mares were in estrus or were not in estrus (had a CL present). Most of the treated mares did not actively seek the stallion and remained below a tease score of 2 and few had a tease score above a 3 indicating that they had altered estrous behavior (red bars, Figure 2.3). Indicating that when teasing either a 3 or above the control mares continued to tease higher whereas the treated mares were on the lower end of the scale.

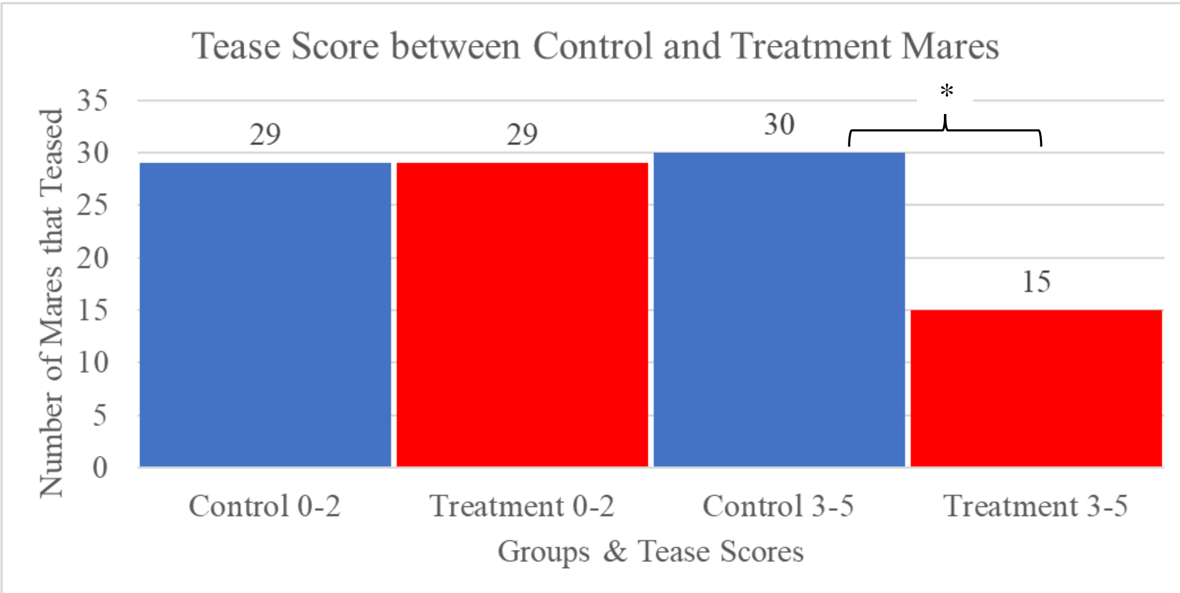

Figure 2.3: Tease Score between Control and Treatment Mares
Tease scores were observed and recorded based on the 20 mares (10 control and 10 treatment mares). Each mare could have teased any score on the 6-point tease scoring system. If the mare teased all 6 scores then she was accounted for in each category. If she didn't tease a 5 and teased a 0 – 4 then those scores were accounted for accordingly. 0-2 tease scores indicated that mares were not sexually receptive and did not interact with the stallion. 3-5 tease scores indicated that mares were sexually receptive towards the stallion and actively teased and urinated. Comparing the groups, the 3-5 scores were significant p-value < 0.0001 between treatment groups.

Ovarian Activity

Overall ovarian activity of the treated mares was minimal compared to that of the control mares p-value < 0.0001 (Figure 2.4). Control mares maintained regular cyclicity including growing follicles, ovulating, and continuing to grow follicles. Sizes of largest follicle in treated mares declined at the beginning of the experiment then remained consistently low and lacked additional development for the duration of the experiment (Figure 2.4). The treatment mare ovaries grew follicles that were on average a 7.0 mm whereas the control mares had follicles that averaged 26.9 mm. None of the treated mares developed a follicle larger than 23 mm or ovulated during the duration of this study, while the control mares ovulated an average of 10.8 times.

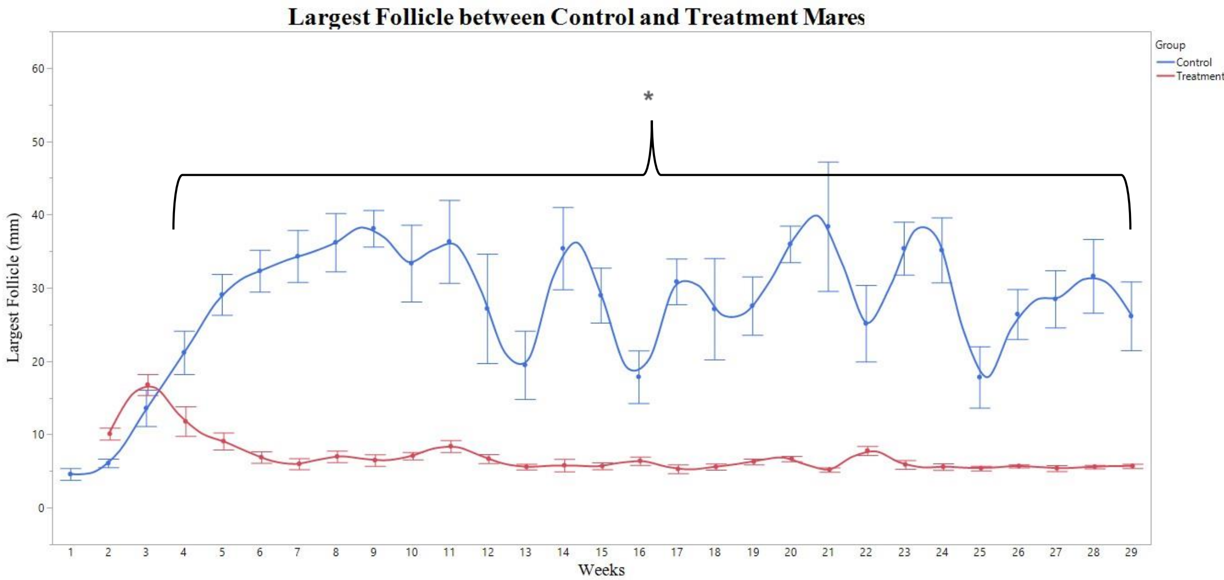

Figure 2.4: Largest Follicle between Control and Treatment Mares
The largest follicles between the control and treatment mares is illustrated and color coded in this graph. Error bars indicate the 1 standard deviation from the mean. The lines represent the average follicle size among mares within their groups (treatment n = 10 and control n = 10). The average largest follicle size between treatment groups is significant with a * p-value < 0.0001.

Progesterone Results

The specific binding was 82 %. Detection limit (95 % of maximum binding) of the assay was 0.1 ng/mL. Intra-assay CV for P_4 was 7.7% and inter-assay CV was 8.3%. The scale that was used to measure the progesterone in the blood serum samples was 0.01 ng/mL to 32 ng/mL. There were some outliers in the control group with very high progesterone levels. Serum progesterone levels in control mares ranged from 67.4 to 0.1 demonstrates cycles while levels in the treatment mares remained below 1ng/mL and developing no functional CL (Figure 2.5).

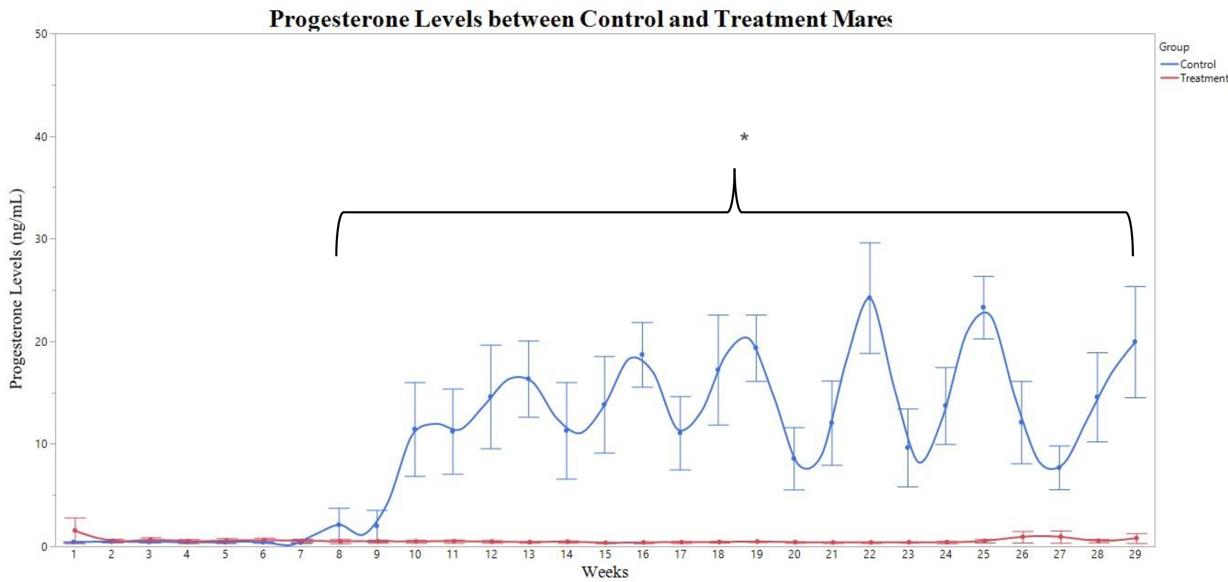

Figure 2.5: Progesterone Levels between Control and Treatment Mares
The circulating progesterone levels between the control and treatment groups was graphed using the same one day per week measurements through the entire experiment. Progesterone levels between the control and treatment mares is illustrated and color coded in this graph. Error bars indicate the 1 standard deviation from the mean. The lines represent the average progesterone levels among mares within their groups (treatment n=10 and control n=10). The average progesterone level between treatment groups is significant with a *p-value < 0.0001.

Discussion

Follicular activity was altered as a result of BMP-15 and GDF-9 combination vaccine. There was no mature follicular activity and a major alteration to ovarian activity occurred. No follicles ovulated in the duration of this project of the mares vaccinated with the combination of BMP-15 and GDF-9 vaccine. Ovaries had minimal activity with follicles measuring 3 – 8 millimeters for the duration of this experiment. The average ovulations between groups was measured by averaging all ovulations over the course of the experiment. The control mares averaged 10.8 ovulations whereas the treatment mares had 0 ovulations across the course of this experiment. The oocyte reserve was theoretically staying the same and was blocked from releasing oocytes into the pool of follicles. GDF-9 and BMP-15 have a major role in follicular development (J. L. Juengel & McNatty, 2005). The same concept of using the growth factors as a blocking spot for the contraceptive to target has been used in deer. For deer it has been researched that vaccinating against GDF-9 alone has been shown to cause a decrease in ovulations (Douglas et al., 2014). As for the sheep, studies have shown that mutations with the BMP-15 and GDF-9 have caused infertility in those ewes (Otsuka et al., 2011).

Progesterone levels were altered in mares that received the combination vaccination. Progesterone levels were analyzed, and results indicated that mares receiving the vaccination had little to no circulating progesterone in their blood (Figure 2.5). Since there wasn't any follicular activity to produce a mature follicle to result in a CL there wasn't any progesterone being produced.

GDF-9 and BMP-15 are key growth factors in follicular development. Studies have shown that GDF-9 is essential in the earlier follicular developmental stages (Otsuka et al., 2011). BMP-15 is involved with the follicle growth and development (Otsuka et al., 2011). By stopping the initial communication between oocytes and development nothing can be recruited to mature and ovulate. Therefore, by blocking GDF-9 and BMP-15 we hypothesize that this has blocked the ovary's ability to communicate to the oocytes in the reserve and there are no oocytes maturing or developing. As more mares cycled in the control group the higher the average progesterone levels were and in the treatment group there wasn't any progesterone.

Estrus behavior was altered in mares that were vaccinated with the BMP-15 and GDF-9 vaccine. The treatment mares tolerated the presence of the stallion and towards the end of the breeding season some mares teased by urinating, but it was more relaxed than a normal mare would tease to a stallion if she was in true estrus. Although some treatment mares accepted the presence of the stallion more willingly than others, their ovaries were nearly identical and not active during the season. The lack of teasing is due to the lack of estrogen that is present in the mares due to the lack of dominant follicles producing estrogen. Without the development and maturation of follicles there is no dominant source of estrogen circulating in the blood to initiate true estrus behavior. Further research is needed to determine the effect of the altered behavior on the wild horse populations. The lack of intense estrus behavior might not have any effect on the herds because from this study most mares still accepted the presence of the stallion they just did not actively tease to him as if they were truly in estrus and sexually receptive. It was also noted that treatment mare behavior changed as the breeding season went along and they began to tease more. There is the potential for learned behavior due to the lack of follicular growth and lack of progesterone.

Changes in the follicular activity, progesterone production and estrus behavior are a huge part of what is to come with future investigations. Further research needs to be done on the duration of the sterilization of the mares that receive the BMP-15 and GDF-9 combination vaccine. Research also is needed on the effect of the lack of estrus behavior on the herds in the wild. The goal is to develop a vaccine that will permanently sterilize mares. In the wild that are in overpopulated areas to help minimize the overgrazing and lack of water to sufficiently fed and water those that live on the allotted BLM land.

Conclusion

In conclusion, this study resulted in mares that did not cycle when vaccinated with a combination BMP-15 and GDF-9 vaccine. Further research is needed to determine the length in which the mares remain sterile and to determine if estrous behavior alteration is reasonable for wild horse herds. Our hypothesis was correct and this immunization vaccine against the two growth factors that normally trigger oocytes to develop into mature oocytes and follicles that ovulate resulted in no ovarian cyclicity in the mares. This vaccination has the potential of becoming a contraceptive for mares in the wild horse herds and a way of sterilizing mares.

Conflict of Interest

We wish to confirm that there is no conflict of interest with this publication and there has been no significant financial support for this work that could have influenced the 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 the authors listed in the manuscript has been approved by all of us.

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CHAPTER 3 – DISCUSSION

Discussion

Successes of the Project

In conclusion of this project, there were many successes that in the end are very exciting and noteworthy for the equine industry. Estrous behavior of the treated mares was decreased by 50% compared to the control mares. This result is impactful and practical because ideally estrous behavior in current bands of wild horses would be minimal due to the mares naturally being in foal 11 months out of the 12-month year. Foaling rates and pregnancy rates of wild horses are close to 100% with the natural selection of wild horses being the most reproductively sound. With less all year around estrous behavior of the vaccinated mares it would put less of a damper on the herd dynamics because it would be normal for the mares to not present and display intense estrous behavior towards the stallion. It is noted that often mares were indifferent towards the stallion with evaluating the behavior of the mares. This behavior would suggest that there would be minimal changes to the herd dynamics due to lack of intense estrous behavior.

Follicular activity was significantly altered in the treated mares compared to the control mares in the previous study (Davis et al., 2018). The BMP-15 average largest follicle size for the year one study was 21.3 millimeters and the GDF-9 mares averaged 27.3 millimeters (Davis et al., 2018). It appears that follicular activity was drastically affected by the BMP-15 and GDF-9 combination vaccine as the average follicle size remained 7.0mm in diameter. There is a possibility that the follicular activity was also significantly increased by more oocytes and follicles entering the atretic stage of follicular development. These atretic oocytes and follicles would never make it to antral or pre-ovulatory size to be seen on the ultrasound. Follicular activity was decreased in the number of antral and pre-ovulatory sized follicles which are the follicles large enough to be seen using ultrasonography. Follicles and oocytes might have entered the follicular pool but due to the lack of signaling of BMP-15 and GDF-9 there was a greater number of oocytes that made it to the atretic stage.

Ovulation rate was the most affected by this contraceptive vaccination and was the primary target of the project. Control mares ovulated 10.8 times comparted to the treated mares that didn't ovulate. The on average 7.0mm sized follicles never ovulated and were eliminated by atresia the follicular fluid remained clear indicating that these follicles were not persistent anovulatory follicles or hemorrhagic follicles. This result is a huge success that has come out of this experiment and meets the hypothesis and goal of the experiment.

Progesterone analysis was processed on all mares for every week that the experiment took place. The progesterone results confirmed that the mares did not ovulate or have corpus luteum present over the course of the experiment. The treated mares remained below 1 ng/mL of circulated progesterone whereas the control mares had a ranging level of progesterone from 64 ng/mL to less than 1.0 ng/mL of circulating progesterone. The cyclicity of the control mares was confirmed by the progesterone that was detected in the blood samples indicating that there were corpus luteum present during the experiment.

The big success of this project is the fact that there were not any ovulations in vaccinated mares. This success will lead to further investigation of this contraceptive vaccination to help control the overpopulation of the wild horses and burros that are on the federal BLM land today. This project has the potential to make a huge impact on the issue that is currently a problem for the BLM with overpopulation of wild horses and burros. I foresee this immunocontraception vaccination continuing to be developed and researched and the public accepting this concept as a more practical and effective way to help control the population compared to current castrating and ovariectomizing methods of sterilizing. The demand for a solution is very high and with the success and exiting results that were determined with this study I think that there will be more research and continuation of this vaccination.

The goal is to make a single vaccination that will cause mares to become sterile. At this point the vaccination will need to be continued to be researched and improved. Various adjuvants will need to be researched to determine the best long-term adjuvant to use. So far, this immunocontraceptives vaccination has proven to be effective in year 1. The current vaccinations including PZP and GonaCon are 1-year contraceptives for mares and this one would add to that list but in hopes of it becoming a longer lasting and permanent vaccination that caused sterilization of the mares. Ideally the mares would continue to build antibodies against BMP-15 and GDF-9 for the rest of her lifetime to maintain sterility.

The reality to the overpopulation of wild horses and burros is that this is going to take many years of vaccinating and managing before we can get a handle on the situation. If we start sterilizing mares that is going to guarantee that the vaccinated mare will not produce a foal. Compared to castrating a stallion where it is likely that an outside stallion will breed the mares and thus the herd continuing to increase in size. After further explanation the public will likely be able to see that the wild horses are suffering and that sterilizing mares is a valid option.

Possibility for Advancement

This vaccination has numerous ways that it can be advanced. Administration of the vaccination can be improved upon and more cost efficient. An option would be to dart the mares from helicopter like the BLM has been doing. Or when captured vaccinate them then but this option is costly from the round up. It has been thought that a feeding system could be put in place where the feeder has a microchip reader and darts the vaccine and microchip into the chest of any horse or burro that comes to the feeder that is not microchipped. The benefit is that since horses are curious they will continue to come back to the feeder and the ones that have already been darted will bring horses that have not been darted. The vaccination would not affect stallions or foals because the target of the vaccination is oocyte specific. Although it would become an issue for fillies that would be darted before they reach puberty because they would be microchipped. If the feeder is high enough off the ground it will not be as big of an issue. Another option would be to make the vaccination in powder form and apply in on feed. There has been research on the variable success associated with the baiting and vaccinating (Sorensen, Beest, & Brook, 2014). Disease transmission among the species that approach the vaccinated feed is high (Sorensen et al., 2014). There is also the thought that other species ingesting the vaccination, but in this case, it is equine specific which is important to note. Another issue with powdered supplementation on feed would be the over consumption by horses and its effect on those horses.

Advancement in the behavior dynamics associated with the vaccinated mares will need monitoring to confirm that no difference in social behavior. Since wild horses have an extremely high pregnancy rate and are pregnant for 9 months out of the year their behavior towards the stallion is more uninterested and combative. Therefore, the mares whom are sterile and not cycling will have the same relative behaviors as pregnant mares.

This project has a lot of potential for further advancement and application. As it evolves and gains interest I think that this will become an exciting topic and a helpful tool in not just the wild horse population but also in other species such as groundhog overpopulation and various wild species populations.

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