Detailed Summary of University-led Research Projects for Improved Fertility Control Tools for Wild Horses

1a. Title: Evaluation of minimally invasive methods of contraception in wild horse and burro mares: tubal ligation and hysteroscopically-guided oviduct papilla laser ablation

1b. Recipient: Oregon State University

1c. Additional Information:

Abstract: In an effort to develop minimally invasive, low risk techniques for contraception and population control in female wild horses and burros, we propose evaluating two procedures, tubal ligation and hysteroscopically-guided laser ablation of the oviduct papilla in standing sedated females.
For tubal ligation, we hypothesize that a flexible endoscope inserted through a small incision in the vaginal vault will allow visualization of each oviduct in pregnant and non-pregnant mares. Use of a diode laser or cautery instrument will allow effective fulguration followed by bloodless sectioning of the oviduct. This procedure should allow successful sterilization of up to 100% of female wild horses and burros gathered in any particular location as a single event.
For the hysteroscopic procedure, we expect to endoscopically visualize each oviduct papilla in standing, sedated, non-pregnant mares. A diode laser will be used to to seal the opening between the oviduct and each uterine horn, thus preventing subsequent fertilization.
The authors feel the proposed procedures will be acceptable to the public because they do not involve major surgery, are expected to have minimal complications while approaching 100% effectiveness, and when applied, are expected to result in a static to decreasing population level. Additionally, tubal ligation is a technique commonly performed in humans.
Fulfilling our objective of developing an acceptable sterilization technique will benefit the public by controlling the population levels of wild horses and burros. In the face of scare feed, drought, or grazing pressures by other herbivores, having some control of the number of wild equids will result in healthier animals and grazing lands.

2a, project 1. Title: Tubo-ovarian ligation via colpotomy as a method for sterilization in mares

2b, project 1. Recipient: University of Kentucky

2c, project 1. Additional Information:

As reviewed in the recent report from the National Research Council [1], female methods of fertility control in equids include surgical ovariectomy, immunocontraceptives directed against the zona pellucida (pZP) or against gonadotropin releasing hormone (GnRH), GnRH antagonists, steroid hormones and intrauterine devices. One important and widely applied technique in humans, tubal ligation, was not addressed in the NRC report. The premise of the current proposal is that techniques for tubo-ovarian ligation can be performed safely, economically and effectively for sterilization of female equids under field conditions. Access to the reproductive tract would be achieved in the standing animal using well-defined sedation / analgesia / local

anesthesia techniques and a surgical approach through the vaginal wall (colpotomy).

Ovariectomy of the mare via colpotomy has been used for many decades as a method for sterilization in domestic horses [2-4]. The technique provides access to the ovary in the standing mare via an incision in the cranial vagina and is routinely performed in the standing animal in domesticated horses. Ovaries are removed, and hemostasis is achieved via the use of an ecraseur which simultaneously cuts and crushes the ovarian pedicle [2-4]. Although effective, the procedure can be accompanied by a high rate of complications (approximately 4% in one study [5]) due primarily to excessive hemorrhage from the ovarian pedicle, and such complications were described in the NRC report [1] as severely limiting application of ovariectomy through a colpotomy approach in addressing the needs for controlling fertility in wild equids. We propose an alternative technique (tubo-ovarian ligation) to induce ovarian necrosis / degeneration secondary to ischemia along with ligation of the oviduct to provide an additional measure to ensure complete loss of fertility in treated mares.

As an alternative approach to ovariectomy in mares, procedures have been described for application of a ligature (nylon zip tie) to the ovarian pedicle in mares via laparoscopy [3]. With this procedure, the ovarian pedicle is ligated via flank laparoscopy, and the ovary undergoes ischemic necrosis or atrophy secondary to a loss of blood supply. Recovery of mares subsequent to the procedure is rapid, and loss of ovarian function is complete [3]. Two other studies have examined the use of zip ties to achieve ligation of the ovarian pedicle for hemostasis with subsequent removal of the ovary by sharp dissection [6, 7]. In the study by Cokelaere et al. [7], standing laparoscopic application of a zip tie was used to achieve hemostasis of the ovarian pedicle with subsequent ovarian excision in 10 mares. Hemostasis was reported to be effective in this method, thereby establishing the use of these devices to effectively control ovarian blood flow. In the second study by Rabelo et al., [6], application of the zip tie was performed through a surgical laparotomy in 12 mares. On the left side, the ovary was excised after application of a zip tie to the ovarian pedicle. On the right side, the zip tie was placed on the ovarian pedicle without removal of the ovary. Mares were examined 15 to 45 days postoperatively for gross and histologic changes in the right ovary which had been ligated *in situ* with the zip tie. The authors reported evidence of atrophy and ischemia of the right ovary subsequent to ligation without evidence of adhesion or abscess formation in the 12 mares examined [6]. The risk of significant hemorrhage from the ovarian pedicle is eliminated using this approach, and we propose to develop a procedure that can be applied in the field in standing mares under sedation / local anesthesia to accomplish simultaneous ligation of the ovarian pedicle and ligation of the oviduct in mares to achieve sterilization via a colpotomy approach for tubo-ovarian ligation in mares.

Nylon (polyamide) cable ties (zip ties or tie raps) have been used in a number of experimental and clinical surgical applications [3, 7-9]. Although not approved medical devices for the horse, nylon cable ties can be heat sterilized and are available in a range of lengths and strengths that lend them to the proposed application in this study. As noted earlier, nylon cable ties have been used to ligate the ovarian pedicle in mares for ovariectomy as well as to induce ischemia and atrophy of the ovary *in situ* [3, 7]. Although long-term follow up to evaluate the biocompatibility of these nylon zip ties was not reported in these studies, there was complete encapsulation of the ovarian stump and zip tie within three to four weeks after application [6].

Nylon zip ties have been used in other species for ligation and hemostasis during ovariohysterectomy without problems associated with the device over time as an implanted foreign body [8]. A number of studies describe laparoscopic ovariectomy in the standing mare under sedation / analgesia and local anesthesia [3, 4, 7, 10]. Field application of laparoscopic surgery in wild horses is impractical due to considerations of equipment cost, technical and surgical skill requirements, as well as the relatively long surgical time and difficulties in appropriate restraint for the procedure in wild equids. Thereby, laparoscopic application of a tubo-ovarian ligature is impractical in a field setting.

The use of vaginal surgical approaches (natural orifice transluminal endoscopic surgery - NOTES) for access to the ovary for ovariectomy have been described in the mare [11, 12] based upon the use of a flexible videoendoscope introduced through a vaginal incision to allow imaging of the ovary and a bipolar vessel sealing device for ovarian removal. Cited advantages to the transvaginal approach include the absence of a skin and body wall incision, rapid healing, less tissue dissection, fewer wound complications as well as an improved post-operative recovery [12]. Again, as with laparoscopic approaches to the ovary, transvaginal endoscopy has relatively large requirements for equipment, technical and surgical skill, surgical time and need for appropriate restraint which limit the application of this approach to the ovary in wild equids.

We propose, however, a two-phase approach to refine the use of colpotomy for tubo-ovarian ligation. In the first year, a standard manual colpotomy approach will be used for ligature placement to demonstrate the feasibility of this procedure to induce ovarian atrophy / tubal ligation. During the second year of the study, we will evaluate a custom designed video-driven instrument which will allow introduction of the ligature through a small vagina port without the need of introducing the operator's hand into the abdomen. This approach will provide the advantages of absence of body wall incision, rapid healing, reduced operative time, reduced wound complications, and reduced risk of hemorrhage from the ovarian pedicle to facilitate tuboovarian ligation in the mare under standing sedation / analgesia.

Because a high proportion of wild horses gathered from BLM – managed lands are likely to be pregnant during the period of July – March when it is possible to work with these animals, the impact of pregnancy on the application of the proposed tubo-ovarian ligation via colpotomy is an important consideration. Ovariectomy or disruption of ovarian function will cause pregnancy loss during the first 70 days of pregnancy due to the loss of ovarian progesterone which is the major progestational support of early pregnancy [13]. In addition, it appears highly likely that the proposed procedure will not be useful in mares during late gestation due to the difficulty in performing a colpotomy in such mares as well as the limited access to the ovaries as the reproductive tract moves cranially and ventrally with increasing gestation.

Ultimately, the application of any contraceptive / sterilization procedure in wild horses must reduce the current level of population growth present in many Herd Management Areas [1]. Although male-directed procedures have been proposed as a means to regulate population growth [1], it is not at all clear that such approaches will effectively limit population growth under field settings. Likewise, the most widely applied contraceptive modality in wild horse mares, porcine zona pellucida vaccination, has been used in more than 4,562 mares in 80 of 179 HMAs since 2004 without significant reductions in population increase

(http://www.blm.gov/wo/st/en/prog/whbprogram/science_and_research/fertility_control.html). Although the reversible contraceptive effect of pZP immunization may have application under some management situations, a permanent sterilization as proposed here may ultimately be a more practical method to control population growth.

Although we anticipate that the proposed tubo-ovarian ligation will be a quick, effective and economical method to sterilize mares, there are a number of areas that remain to be assessed relative to application in wild horses. First, complete ablation of ovarian function as proposed here will affect the behavior, social structure and herd makeup in bands of wild horses; however, the same is true for all of the proposed or existing methods for reproductive control in the mare [1]. In the absence of normal ovarian cyclicity, mares will continue to demonstrate sporadic estrous behavior likely due to steroid production by the adrenal gland [14]. Continued estrous behavior in these mares may play an important role in retaining the harem structure of the band [1]. Second, application of the technique will likely be limited to nonpregnant mares or mares during the first half of pregnancy. Third, operator and animal safety is a concern using this approach. The NAS report [1] precluded ovariectomy in wild horse mares due to concerns about hemorrhage and peritoneal infection. We believe that the procedure proposed here eliminates the risk associated with hemorrhage during ovariectomy and thereby problems with peritoneal infections as well. Operator safety and training will also be an important consideration. Although wild horse mares have been ovariectomized under field conditions via a colpotomy approach, restraint and sedation present challenges that are not encountered with domesticated mares. Ultimately, we estimate total procedure time using this technique to be less than 7 to 10 minutes. The further development of a videoendoscopic approach for application of the tubo-ovarian ligature should further reduce the time required for the procedure as well as reduce the risk to the operator by allowing a more remote approach to the ovaries. Further refinements in restraint systems and sedation / analgesia protocols are also likely to greatly enhance animal and operator safety with this procedure.

Attempts to affect population control in wild mammals have presented a real and persistent challenge across a wide range of ecosystems and animal types. Reproduction remains a very robust process and efforts to date to manage populations of wild mammals have been only variably successful. The proposed technique of tubo-ovarian ligation is a simple, direct and permanent method to prevent reproduction in the mare. The proposed application would have direct impacts on both wild populations of horses as well as domestic horses where a rapid, safe and inexpensive method to eliminate reproductive behavior may be desirable for many mare owners.

Goals and Objectives:

The overall goal of this proposal is to develop methodology for the safe, economical and effective sterilization of mares via colpotomy (vaginal incision) to achieve: 1) ovarian necrosis / atrophy via application of a ligature to the ovarian pedicle and 2) simultaneous sterilization via tubal ligation (i.e., tubo-ovarian ligation).

***Objectives*:**

1. Determine the effectiveness of a custom-designed instrument for placement of a polyamide (nylon) cable tie (zip tie) around the ovarian pedicle and oviduct of mares via colpotomy for tubo-ovarian ligation. The procedure, conducted in the standing animal under sedation and local anesthesia, is expected to induce permanent sterilization of treated mares.
2. To assess post-operative complications of the procedure in mares and the effects on the health

of mares.
3. To determine long-term effects on the reproductive tract, and the overall health of mares and the fertility of mares undergoing the procedure.
4. To assess the feasibility of these procedures in pregnant mares.

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3a. Title: Functional assessment of ovariectomy (spaying) via colpotomy of wild mares as an acceptable method of contraception and wild horse population control

3b. Recipient: Oregon State University

3c. Additional Information

Abstract: Department of Interior Secretary Ken Salazar recently put forth new proposals for the management of wild horses and burros, including new strategies aimed at balancing wild horse and burro population growth with public adoption demand. Proposed strategies include slowing population growth rates of wild horses and burros on public rangelands through the aggressive use of fertility control. It is our hypothesis that ovariectomy via vaginal colpotomy can be safely and effectively performed on wild mares that have been selected for non-breeding status. Those individuals could then be returned to the range to live out their natural lives without individually contributing to population growth. The proposed research effort is based on recent pilot studies that have suggested the potential for surgery-related health complications from ovariectomy in adult female horses is low (near 1%). When evaluating options for field techniques, spaying (ovariectomizing) mares as a population control method is not recommended unless it can be performed in a safe, practical, and effective manner. This project proposes to conduct a large scope investigation of the safety and practicality of spaying mares as a tool for wild horse population control. The results of this study will provide standardized, baseline outcomes for this surgical procedure which can be directly compared to other less invasive procedures being conducted and evaluated by the same research team.

Purpose, Objectives, and Relevance

A. Why the project is needed by the applicant:

This project will demonstrate that ovariectomy in wild mares is a procedure which can be safely and efficiently applied in the field to permanently sterilize wild horses. Successfully performed, it should be 100% effective. The need for permanent sterilization has become evident as the reproductive rate in wild horses exceeds the ability of the BLM to effectively and humanely manage horses (provide adequate feed and water) while protecting the ecology of the range. As the number of horses in holding facilities continue to escalate, the need for effective, practical, and economical techniques to control the reproductive rate in the wild horse populations is necessary.

B. Applicant's objectives:

Our objectives are to utilize a proven field technique for ovariectomy (spay) and apply it in a trial that will evaluate the feasibility of utilizing surgery as a management tool in controlling the growth rate of wild horse herds. Since this procedure has been used as an effective tool for wild horse management on Sheldon National Wildlife Refuge, we propose that the BLM can also utilize spaying of wild mares to address the overpopulation issue on public lands. The objective of this trial is to show that, despite the risks associated with this surgery, the complication rate is very low and indeed very acceptable and that horses undergoing this procedure do not suffer unnecessary discomfort from the procedure.

C. How do the objectives support the applicant's mission:

Our objectives are to develop a practical and cost-effective technique for permanent sterilization of wild mares. This and other proposed techniques will support our mission of seeking ways to alleviate excessive wild horse population growth rate. We think that mare management holds the key to population control. A concise procedure that is 100% effective and permanent is not only humane to the horses but of great benefit to the public by helping preserve the range in a healthy condition so that wildlife and plant biodiversity is not threatened by equine over population. Multiple uses of public lands maximize benefits to animals and the public.

4a. Title: Re-immunization of Free-Ranging Horses with GonaCon Immunological Vaccine: Effects on Reproduction, Safety, and Population Performance

4b. Recipient: Colorado State University

4c. Additional Information:

BACKGROUND

1. Re-immunization

In many areas of the western United States, overabundant and rapidly expanding populations of feral horses (*Equus caballus*) pose a significant dilemma for natural resource managers. The Wild Free-Roaming Horses and Burros Act of 1971(P.L.92-195) provided protection for feral horses and burros (*Equus asinus*) on most federal lands and established guidance for their management as a wildland species (Wagner 1983). There is, however, wide-spread concern among state, federal, and private land management agencies that unregulated feral horse populations are severely altering native plant communities and limiting the abundance and diversity of habitat resources allocated for native wildlife and other domestic livestock species.

Current population control methods such as utilizing periodic roundups and adoption or sale of excess animals, or maintaining excess feral horses in long-term holding facilities are expensive, resource intensive, and unsustainable. Clearly, more efficient, cost effective, and humane approaches to reducing feral horse densities on public lands are needed. Controlling the fertility of female horses offers a potential non-lethal alternative to conventional methods (National Research Council 2013).

A promising immunological approach to contraception in feral horses involves immunization against the neuropeptide gonadotropin releasing hormone (GnRH). Scientists at the National Wildlife Research Center (NWRC) have conjugated synthetic GnRH peptides to a highly immunogenic carrier protein that, when combined with a potent adjuvant, stimulates the host's immune system to produce antibodies that bind to endogenous GnRH. This, in turn, prevents synthesis and secretion of important downstream reproductive hormones necessary for

reproduction. Animals generally return to fertility as antibodies concentrations decline (Powers et al. 2011).

Multiple years of infertility have been achieved in captive and free-ranging wild ungulates with a single inoculation with the GnRH-based vaccine, known as GonaCon. This vaccines has been experimentally tested and found to provide multiple years of infertility after a single application in white-tailed deer (*Odocoileus virginianus)(* Miller et al. 2008, Gionfriddo et al. 2011*a*), bison (*Bison bison)(* Miller et al. 2004), elk (*Cervus elaphus)(* Killian et al. 2009, Powers et al. 2011, 2014), wild pig (*Sus scrofa)(* Massei et al. 2012), and feral horses (Killian et al. 2008, Gray et al. 2010, Baker et al. 2013). However, multiple years of infertility are only experienced in a fraction of vaccinated animals. In free-ranging elk, there was approximately a 90% treatment effect the first year after vaccination but that dropped to 50% by the second year and by the third year of the study, there was no measureable response (Powers et al. 2014). Similarly, during the first 3 years of our current investigation in feral horses at THRO, we observed a 25-35% decrease in foaling in treated versus control mares for the first and second years of the study but no effect by year three (Baker et al. 2013).

Repeat vaccinations generally result in a more profound and longer-lasting antibody production due to the anamnestic response (Tizard 1982). Therefore, we expect longer- lasting contraceptive effects in re-vaccinated mares. The single-injection GonaCon vaccine is unique in that the formulation initiates high antibody titers that remain elevated in some applications; however, to our knowledge, no research has been conducted to evaluate booster doses of this vaccine in any mammalian species.

Booster immunizations using a variety of GnRH vaccines in domestic horses have been shown to improve contraceptive efficacy and to suppress behavioral and physiological estrus (Garza et al.1986, Elhay et al. 2007, Botha et al 2008). However, these GnRH vaccines differ from GonaCon in that they incorporate different protein carrier molecules and adjuvants, and are formulated for short duration (< 1 yr.) contraceptive effectiveness that is generally achieved by using a primary immunization followed 35 days later by a booster inoculation.

While a single vaccination is often preferred from a management perspective, GonaCon vaccine may prove to be more effective if repeat vaccinations are delivered on a periodic basis. Efficacy data collected from 25 mares treated with single application of GonaCon in 2009, at Theodore Roosevelt National Park (THRO) revealed a moderate 2-year decline of approximately 30% in foaling rates, with all mares regaining fertility by three years post-primary vaccination treatment (Baker et al. 2013). Surprisingly, re-vaccination of these same mares in the fall 2013 (four years post-primary vaccination) has resulted to date, in complete infertility during the 2015 foaling season (the first season to expect a re-vaccination effect on fertility). Clearly, these results are both statistically and biologically significant, as well as encouraging from a fertility control perspective.

If these results persist over time and these mares remain infertile, it would lend support to our hypothesis that re-vaccination with GonaCon, even four years post-primary vaccination produces a strong anamnestic response in horses that stimulates anti-GnRH antibodies and suppresses fertility. At present, however, it is premature to predict how many of these re-vaccinated mares failed to conceive during the 2014 breeding season and will not foal or regain fertility during 2015 and beyond. It is possible that the booster vaccination simply delayed the estrous cycle in these mares, which could result in foals being born later in the foaling season.

While these findings are tentative and inconclusive, they suggest that repeat vaccinations are likely needed to achieve high efficacy of GonaCon vaccine in free-ranging horses and these

effects have not been investigated or determined. Thus, our proposed research offers a unique opportunity to address this question at THRO and will have relevance, not only to feral horses, but also to other wild ungulates that have been treated with a single treatment of GonaCon vaccine. Our proposed research will begin to define the vaccination schedule needed to maintain infertility in free-ranging horses and whether or not long-term or permanent sterility is a possible outcome. We will investigate the safety and efficacy of a repeat vaccination under the hypothesis that this vaccine will be more efficacious and longer-lasting than the original primary immunization.

2. Remote Dart Delivery

Fundamental to practical field application of GonaCon vaccine in free-ranging horses is a safe, reliable, and effective method of administering a single dose of the vaccine to free-ranging horses by means of a syringe dart. Many contraceptive agents have been successfully applied via syringe dart or biodegradable implant to an assortment of wild ungulate species including white-tailed deer (Turner et al. 1992, Jacobsen et al. 1995, DeNicola et al. 1997), elk (Shideler et al. 2002, Baker et al. 2005), feral horses (Kirkpatrick et al.1990, Roelle and Ransom 2009), and elephants (*Loxodonta Africana*) (Delsink et al. 2002). However, to our knowledge, evaluation of remotely-delivered GonaCon vaccine is limited to one field investigation with white-tailed deer (DeNicola unpublished data). Although dart performance in this study was less than expected, it provided important basic information regarding optimum dart configuration and delivery ballistics. Using this preliminary data, technicians at Pneu-Dart, Inc. developed a prototype dart configuration for delivering this highly viscous vaccine formulation to free-ranging horses.

We tested this GonaCon-specific dart delivery system with captive feral horses at the 2013 scheduled roundup at THRO. Eleven adult mares (2-4 years of age), that had not been previously vaccinated, were held in small paddocks and remotely darted in the biceps femoris muscle with 2 ml (2000 µg) of GonaCon vaccine. All darts were weighed (± 0.01g) before and after injection to determine the precise dose delivered. Darting distance varied from 10-15 m. Nine out of 11 darts delivered, on average, 95% of the GonaCon vaccine formulation. Two darts failed to discharge possibly due to low muzzle velocity. All darts appeared to dispense the vaccine deep into the muscle mass and none of the darts were observed to bounce without penetration, partially discharge, blow-out, or show evidence of subcutaneous delivery of the vaccine. The two horses in which the darts failed to discharge were subsequently re-treated and the second darts successfully delivered a full dose. With 85% of the 2015 foaling season complete, 7/11 (63%) of these mares have not foaled. In contrast, only 16% of the untreated mares have not foaled to date. A dependable dart delivery system for administering GonCon remotely to free-ranging horses is critical to the determination of an optimum re-vaccination schedule in our proposed study. If successful, this technology will potentially provide resource managers with an alternative strategy for managing this feral horse population.

3. Biological Side-Effects

Evaluation of the biological side-effects of GonaCon vaccine treatments have been reported for numerous wild ungulate species including white-tailed deer (Curtis et al. 2008, Gionfriddo et al. 2011*b*), elk (Powers et al. 2011, 2012, 2014), bison (Miller et al. 2004) and feral horses (Baker et al. 2013). Results from these investigations generally conclude that GonaCon does not cause serious adverse effects on general health, body condition, existing

pregnancy, neonatal health, major organ systems, or fertility of male and female offspring of females treated during pregnancy.

Granulomatous intramuscular injection-site lesions, that occasionally break and drain as abscesses, are the only adverse effect of vaccination consistently reported in these studies. The formation of these injection site lesions may be necessary for stimulation of a strong immune response and infertility. GonaCon vaccine contains AdjuVac; a water-in-oil based adjuvant developed from a USDA approved Johnes disease vaccine called Myocopar^{TM} (Fort Dodge Animal Health). AdjuVac contains killed *Mycobacterium avium,* which is needed to induce a rapid, strong, and sustained contraceptive response (Miller et al. 2008*a,* Perry et al. 2008). This combination of water - in- oil emulsion and killed mycobacteria results in a highly potent adjuvant that stimulates both humoral and cellular immunity (Warren et al. 1986).

Vaccines, like GonaCon, that contain mycobacteria may induce strong immune responses because of the formation of a repository or depot at the injection site (Fukanoki et al. 2000). In response to the presence of the depot, a granuloma forms as the immune system attempts to isolate the foreign material. The continued existence of this depot, which initiates a chronic inflammatory response, likely provides a long-term source of antigen stimulation and persistent antibody production. We speculate that this is the mechanism by which a single vaccination can provide multiple years of infertility in a portion of the population in many species that have been studied.

However, even with this prolonged antigenic stimulation, the immune response from a single vaccination does not consistently provide multiple years of infertility in all or even a high proportion of animals (Powers et al. 2014, Baker et al. 2013). In all studies, where post-mortem examinations were performed, prevalence of injection-site inflammation and granulomas were present but in some species, such as white-tailed deer and elk, they were not apparent antemortem (Curtis et al. 2008, Powers et al. 2011, Gionfriddo et al. 2011*b*).

In contrast to these species, injection site reactions in feral horses, following GonaCon vaccination at THRO, are readily observable as subcutaneous swellings. In past studies at THRO (2009-2013), all injection site reactions appeared to be confined to the general gluteus muscle where the vaccine was first hand-injected. Reactions to the vaccine were first observed 30 days post-treatment in 17.2% (5/29) of mares and by the second breeding season, 79.3% (23/29) of treated females showed some evidence of inflammation or swelling at the injection site. Saline control mares displayed no evidence of injection site reactions. Swellings of various sizes (marble to baseball size) were most common, followed by nodules, and rarely a draining abscess. Most of these reactions were observable for three years post-treatment, then began to resolve and become less visible by year 4 (many that could not be visually observed were still manually palpable at the 2013 roundup).

However, similar to other studies where injection site reactions have been evaluated, we did not observe any clinical evidence of lameness, impaired mobility, depression, or decreased health or fitness in any animal that was associated with GonaCon vaccine treatment. While results from the above investigations are generally consistent relative to the effects of GonaCon-induced injection site reactions, they are also limited to the consequences of a single vaccination usually delivered by hand-injection.

At the 2013 THRO round-up, GonaCon –treated mares were re-vaccinated, four years post-primary vaccination, with a booster dose on the opposite side in the biceps femoris muscle. This investigation is in progress but thus far, injection site reactions appear to be less apparent than those observed following the 2009 vaccination (Baker et al. unpublished). At this time, the

cumulative effects of re-vaccination are unknown and the potential for more intense immune reactions with additional doses of this vaccine delivered by syringe dart is a consideration (Broderson 1989, Roelle and Ransom 2009).

4. Behavioral Side-Effects

Behavioral side-effects of GonaCon vaccination in wild ungulates have not been extensively investigated (Gray et al. 2010, Baker et al. 2012, Ransom et al. 2014). Given the physiological mechanism of action, GonaCon vaccine has the potential to suppress fertility and diminish the reproductive behaviors typically associated with estrus. However, in GonaCon-vaccinated female elk (Powers et al. 2011) and free-ranging horses (Gray et al. 2010, Baker et al. 2012, Ransom et al. 2014) such behaviors were maintained throughout the first breeding season after immunization and were not different from untreated females.

In a previous study at THRO during 2009-2010, daily activity patterns, social interactions, and reproductive behaviors were similar for GonaCon treated and control mares (Baker et al. 2012, Ransom et al. 2014). But, since GonaCon only prevented conception in 50% of treated mares (n = 28), behavioral observations were limited to only 14 infertile females. Thus, inferences to free-ranging feral horse populations are not definitive and deserve further investigation prior to use in management applications.

In an attempt to further our understanding of the behavioral side-effects GonaCon vaccine, we conducted behavioral observations during the first breeding season following re-vaccination of these same mares at THRO in 2013. We measured the effects of this vaccine on sociosexual behavior, harem dynamics, and activity budgets of treated (n = 25) and control (n =25) horses. To date (July 20 2015), none of the re-vaccinated mares have foaled, whereas 84% (21/25) of the control mares have done so. As a result of higher vaccine efficacy in treated mares, our sample size increased by 44% and offered a more rigorous quantitative investigation into potential effects of GonaCon treatment on feral horse behaviors.

5. Population Modeling

We will integrate contraceptive efficacy and population monitoring data at THRO to estimate parameters and unobserved states in a Bayesian hierarchal model (Dulberger et al. 2010, Monello et al. 2014, Hobbs and Hooten 2015, Hobbs et al. 2015, Rahio et al. in review).We will use the model to evaluate the population-level effects of GonaCon on the free-ranging horse population at THRO. We will forecast the consequences of alternative contraceptive strategies on population performance with rigorous evaluation of uncertainty. There is an urgent need to extend studies of efficacy of individuals to populations (Ransom et at. 2014). A key extension of our experimental research is to determine the effects of different GonaCon delivery regimes on the growth rate of the THRO population.

OBJECTIVES:

The primary objectives of this research are:

a) to begin to determine the optimum and most effective re-vaccination schedule with GonaCon vaccine for suppressing reproductive rates in free-ranging horses, the duration of effectiveness, and the return to fertility following treatment.

b) to determine the safety and physiological side-effects (if any) in feral horses following re-vaccination with GonaCon including visual assessment of general health, body condition, injection site reactions, effects on current pregnancy, and neonatal health and survival.

c) to determine the effects of GonaCon vaccination on the behavioral side-effects (if any) in free-ranging horses including quantitative assessment of the effects on daily activity patterns and social interactions.

d) to develop and test a safe and effective dart configuration and injection system for remotely administering GonaCon vaccine to free-ranging horses by means of a syringe dart.

e) to develop a Bayesian model to forecast the consequences of different GonaCon vaccine treatments on feral horse population dynamics at THRO.

HYPOTHESIS:

H1: Female feral horses re-vaccinated with GonaCon will show significantly ($P \leq 0.05$) lower reproductive (yearly pregnancy and foaling) rates than non-treated control mares and contraceptive efficacy of re-vaccinated mares will be greater and longer lasting than that observed following the initial immunization.

Rationale: An immune response is a physiologic reaction to a foreign substance or antigen; especially one mediated by lymphocytes and involving recognition of antigens by specific antibodies or previously sensitized lymphocytes. Vaccines rely on the anamnestic response for optimal function. This response is a renewed rapid production of antibodies on the second (subsequent) encounter with the same antigen. This reaction is possible through memory cells that store information regarding the recognition of an antigen based upon previous exposure. Booster or repeat vaccinations generally result in a more rapid and stronger immune reaction to a second inoculation with the same antigen (Tizard 1982). However, the optimum re-vaccination schedule for GonaCon vaccine in feral horses or any other ungulate species has not yet been investigated or determined.

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5a. Title: The Effect of Immunization against Oocyte Specific Growth Factors in Mares

5b. Recipient: Colorado State University

5c. Additional Information:

As the population of wild horses and burros continues to increase, it is evident that reproductive control will be the favored mechanism employed to curtail increasing numbers while allowing for herd maintenance on public lands. Reproduction in all mammals including horses and burros depends on an adequate number of healthy oocytes (eggs) present in primordial follicles within the ovaries. The total number of oocytes contained in the ovaries is established either before or shortly after birth. Follicular recruitment and growth is constant and irreversible. Although the mechanism that initiates this metered process has not been completely elucidated, it is known that communication by the oocyte is critical. Additionally, 2 key proteins produced exclusively by the oocyte to regulate this have been identified. To date their *exact* function is unknown, but their dysregulation has resulted in premature oocyte depletion in other species. It is our working hypothesis that vaccination against these proteins either alone or in combination will result in permanent sterility through premature oocyte depletion. The depletion of oocytes may occur by simply causing them all to become atretic prematurely and/or accelerating the process so that after a single season the mares and jennies have depleted their oocyte reserves. To test this hypothesis, we propose to vaccinate mares against the proteins and tracking their sexual behavior, follicular growth, hormonal profile and ultimately total oocyte count over a two-year period.

Goals

Our long-term goal is to develop a vaccine that can cause permanent sterility after a single dose.

Objectives:

The objective of this work is to determine the effects of immunization against the specified proteins on ovarian function in horses.

Hypothesis:

We hypothesize that blocking follicular growth at the primary stage through vaccination against the study proteins will prevent ovulation and cause an increase in the number of follicles that are recruited to grow. This will increase follicular turnover, and as a consequence cause premature oocyte depletion and permanent sterility.

Successful reproduction in mammals depends on an adequate number of healthy oocytes (eggs) present in primordial follicles within the ovaries. The total number of oocytes contained in the ovaries is established either before or shortly after birth (Eckery et al., 1996; Peters et al., 1976; Sawyer et al., 2001; Tingen et al. 2009). Each primordial follicle consists of an oocyte surrounded by a few somatic (i.e. granulosa) cells. Every day a certain number of primordial follicles begin to grow. Growth is evidenced by an increase in the size of the oocyte and proliferation of the granulosa cells. The initiation of growth is a committed step and the follicles cannot return to a non-growing state. Consequently, nearly all follicles end up dying at some stage with <0.1% going on to ovulation. The entry of follicles into the growth phase, follicular turn over, is tightly controlled to ensure that an adequate number of follicles is available throughout the reproductive life of the animal (Monniaux et al., 2014; Reddy et al., 2010). Consequently, there is a continual non-renewable loss of the finite supply of oocytes throughout puberty and adult life; which in some species eventually leads to complete depletion of the oocytes (e.g. menopause in humans) and sterility. Damage to this finite supply of oocytes, either through genetic mutations (Di Pasquale et al., 2004) or exposure to harmful substances (Hoyer et al., 2014) can also cause permanent sterility.

The physiological mechanisms controlling follicular growth and the number of oocytes released at ovulation involve a complex exchange of systemic (e.g. endocrine) signals between various organs and the ovaries, and a local exchange of molecules between the oocyte and its surrounding somatic cells within the ovaries (Binelli and Murphy, 2010; McNatty et al., 2007b; Peters et al, 1976). Communication between these two cells types is crucial for the survival and growth of the follicles (Gilchrist et al., 2004; Matzuk et al., 2002). Significant discoveries have shown that the oocyte itself produces two key regulatory growth factors that are essential for regulating follicular growth and ovulation rate (Cong et al., 1996; Galloway et al., 2000). These discoveries also led to a paradigm shift in recognizing that the oocyte acts as the control center of the follicle and plays a major role in its own growth and ovulation.

Both of these growth factors have been shown to be critical for early follicular growth in several species (Eckery et al., 2002; Juengel et al., 2002; Juengel and McNatty, 2005; Shimasaki et al., 2004). It has also been shown that these two oocyte-specific growth factors cooperate in a species-specific way to regulate maturation of the oocyte and communicate with the adjacent granulosa cells (Lin et al., 2012). In sheep, it has been shown that animals that have double copy mutations in the genes encoding either of the proteins are sterile, but otherwise healthy. In these animals, follicles do not progress beyond the first, or primary, stage of growth. Interestingly, in animals that have only a single copy mutation in either of these genes, and thus produce essentially only half the amount of protein, ovulation rate is increased (Galloway et al., 2000; McNatty et al., 2005). Mutations in the proteins have also been found in women with premature ovarian failure (Di Pasquale et al., 2004; Pouresmaeili and Fazeli, 2014).

Immunization

In a series of experiments conducted in sheep, it was shown that ewes could be made infertile after immunization against either of the study proteins (McNatty et al., 2007a). Moreover,

specific regions of each growth factor were identified that were important for the biological activity of the respective proteins. This enabled the production of effective peptide vaccines that were specific to those regions. The homologous regions were identified in cows and sheep, and found to have 100% amino acid sequence identity to the respective regions in sheep. In cows, vaccination against these peptide regions showed variable results, where in some animals reproductive cycles were suppressed and in others there was an increase in ovulation rate (Juengel et al., 2009). In deer (Eckery et al., 2014), animals vaccinated against one of the proteins were not made infertile, rather they became more fecund. This may have occurred because the biological activity was only partially blocked and caused an increase of ovulation rate similar to the effect in sheep that have a single copy mutation. Deer vaccinated against the other protein were made more fecund in the first year, but were made infertile in years 2 and 3. The effects observed in the first year could have been because of the timing of vaccination in relation to onset of breeding. Regardless, it appears that the first of the proteins was only partially inhibited during the first breeding season. Results from all of these studies demonstrate that vaccination against the proteins has the potential to control fertility in a range of species. Further research will be required to determine the appropriate timing for administering the vaccine and the longevity of effect. The genes for both proteins have been identified in the horse (Wade et al., 2009) and shown to be expressed in oocytes (unpublished results). Therefore, it is likely that these growth factors have similar functions in horses.

There are two ways that can be considered to cause oocyte depletion. The first would be to use an agent to directly kill the primordial follicles. A second way would be to trigger a mass activation (growth) of the primordial follicles. Because the initiation of primordial follicular growth is a committed step, this would result in all the follicles dying prematurely, leading to oocyte depletion. We hypothesize that blocking follicular growth at the primary stage through vaccination will prevent ovulation and cause an increase in the number of follicles that are recruited to grow. This will increase follicular turnover, and as a consequence cause premature oocyte depletion and permanent sterility.

Objective: The objective of this work is to determine the effects of immunization against two growth factor proteins on ovarian function in horses.

Our long-term goal is to develop a vaccine that can cause permanent sterility after a single dose – one hit, permanent sterility.

Expected results: We expect that ovarian follicles won't grow past the primary stage. Therefore, treated mares will not show signs of behavioral estrus nor ovulate. After the second year, we expect that the number of primordial follicles will be decreased. If ovulation rate is increased, then it is still unlikely that any foals would be produced if a mare was bred, because the uterus of a horse can sustain only a single fetus. Both proteins are only known to affect follicular growth and ovulation rate, therefore, there is no evidence or reason to believe that immunization against these oocyte-specific growth factors would have any effect on pregnancy or fetal viability if a vaccine was administered to a pregnant mare.

6a. Title: Electrospun delivery to enhance the effectiveness of immunocontraception strategies in equids

6b. Recipient: Ohio State University

6c. Additional Information:

A: We seek the support needed to develop an electrospun technology that can allow long-term, 'burst' delivery of porcine zona pellucida (PZP) vaccines to the intramuscular environment of horses and burros to result in prolonged suppression of reproduction. For large-scale application, we foresee the use of standard procedures that call for free roaming horses to be captured in the field and processed through stock chutes for aging, at which time the implants will be inserted by trocar.

B: To carry out parallel *in vitro* and *in vivo* experiments to examine the potential of electrospun vehicles as immunocontraceptive carriers. An electrospun "universal delivery vehicle" will be developed to provide sustained release of effective levels of porcine zona pellucida (PZP) for immunocontraception over periods of at least three years. By careful design, fabrication and testing of two different electrospun designs in both a rabbit model and in domestic horses, we will create a comprehensive evaluation of this novel method of delivery.

C: To reduce population pressure on public lands, horse immunocontraception has largely focused on the use of PZP in free-roaming wild populations. The vaccine appears to act by stimulating anti-PZP antibodies that bind to the surface of the ovulated egg, preventing sperm attachment. While performance has been satisfactory, recent results have been associated with contraceptive efficiencies that are considerably less than 100%. The basis for this is unknown but is believed to be in part caused by delivery methods that require substantial heating during polymer vehicle fabrication, expose PZP to enzymatic fluids prior to entry into the bloodstream and allow *gradual* – not burst – release. Gradual release can potentially desensitize the immune system to the presence of PZP, resulting in inferior production of anti-PZP antibodies. Thus, an ideal delivery method would allow release of PZP in "bursts" at pre-determined intervals to assure constant immune stimulation.

Significance of the Proposed Research: To reduce population pressure on public lands, horse immunocontraception has largely focused on the use of porcine zona pellucida (PZP) in free-roaming wild populations. The vaccine acts by stimulating anti-PZP antibodies that bind to the surface of the ovulated egg, preventing sperm attachment. While performance has been satisfactory, recent results have been associated with contraceptive efficiencies that are considerably less than 100%. The basis for this is unknown but is believed to be in part caused by delivery methods that require (1) substantial heating during polymer vehicle fabrication, potentially impairing vaccine function and (2) allow gradual – not burst – release. Gradual release can potentially desensitize the immune system to the presence of PZP, resulting in inferior production of anti-PZP antibodies and subsequent decreases in efficacy. Thus, the ideal delivery method would allow release of PZP in "bursts" at pre-determined intervals to assure constant immune stimulation and maintenance of protective anti-ZP antibody titers.

Applicant objectives: An electrospun "universal delivery vehicle" will be developed providing release of PZP effective in achieving convincing immunocontraception in a rabbit model. This will then be followed by a 2-year, equine-based study beginning in Year 2.

Mission and benefits to the public: The generation of an electrospun technology allowing long-term, 'burst' delivery of PZP to the intramuscular environment of horses and burros to result in prolonged reproductive suppression.

Permanent sterilization of even young animals would greatly reduce population pressure on public lands.

7a. Title: The use of membrane disrupting peptide / peptoid LHRH conjugates to control wild

horse and burro populations

7b. Recipient: Louisiana State University

7c. Additional Information:

Despite efforts since 1978 to develop an effective contraceptive agent for wild horses and burros, management of these populations in the western United States continues to be a serious problem. As of February 2013, the Bureau of Land Management (BLM) estimates that 40,605 wild horses and burros are roaming on BLM managed rangelands in 10 western states. Wild horses and burros have few natural predators and their herd sizes can double every four years, creating an unsustainable herd population. Currently these herd populations are managed by removal and relocation of thousands of animals to private pastures or corrals. The BLM estimates that the appropriate management level, that is the number of wild horses and burros that can exist in balance with public rangeland resources and uses, is approximately 26,677. With the feral population doubling every four years, the number of excess animals is rapidly exceeding space available for housing in private pastures and corrals. Therefore, new contraception techniques are needed to temporarily or permanently sterilize wild horses and burros in the field to control herd populations.

The proposed research is a multidisciplinary effort aimed at developing novel drugs to control the wild horse and burro populations. In recent years, we have developed several types of drugs consisting of conjugates of membrane disrupting peptides (such as Phor 21) with luteinizing hormone releasing hormone (LHRH). These drugs (such as LHRH-Phor 21 conjugate) effectively target, bind to and destroy prostate, testicular, breast and ovarian cancer cells, as well as testicular and ovarian cells that control reproduction. LHRH targets the cell and delivers Phor 21 to the cancer cell or the reproductive cell in the testes or ovary and destroys it. Preliminary experiments suggest that administration of this drug by a slow-release delivery system will destroy the cells that control spermatogenesis in the male and follicle growth, oocyte development, ovulation and cyclicity in the female. Our preliminary results also show that LHRH-Phor 21 targets and destroys gonadotropic cells in the pituitary gland. This indicates that cessation of reproductive activity is the result of both central control at the level of the pituitary gland and on receptor binding cells in both male and female gonads. We will also assess the effect our drugs have on pregnant mares, both in early gestation and late gestation.

Membrane disrupting peptides are subject to degradation in animal systems by peptidases, reducing their efficacy. Recently, we demonstrated that the peptoid versions of membrane disrupting peptides are just as active as their peptide counterparts against antibiotic resistant bacteria. Where peptides have the constituent amino acid side chains on the alpha carbon, in peptoids, we move the amino acid side chain to the alpha nitrogen. This renders peptidases ineffective against peptoids, extending their efficacy in animal systems. This project will broaden the scope and usefulness of membrane disrupting peptide and membrane disrupting peptoid conjugates as valuable tools in the reproductive control of animals with an emphasis on feral animal populations. If successful, the use of these conjugates could be extended to control other feral animal populations, such as swine which are damaging public range lands,

forests and national parks. The conjugates can also be used to control captive exotic animal populations not destined for genetic conservation.

The logistics of the proposed procedure as applied to wild horses or burros are greatly simplified by our choice of drug delivery. We are proposing to use an injectable peptide gel vehicle to deliver the drug on a time-release basis to the animals. The vehicles are easily synthesized in the lab and can be manipulated to fine tune the drug release rate to an acceptable level. The peptide gels are harmless to the animals and will break down over time. The rate at which the gel breaks down can also be manipulated to achieve a desirable in vivo lifetime. The drug-gel combination can be injected via syringe, either subcutaneously (SQ) or intramuscularly (IM) while the animal is in a chute. It may also be possible to deliver the drug via dart gun. With either type of administration, no pre-operative procedures, restraints (other than a chute), or sedation are required. High animal welfare and practitioner safety are maintained at all times.

Background and Significance/Preliminary Studies

As stated previously, the wild horse and burro population is quickly becoming unmanageable, creating an urgent need to control their reproduction. The research outlined in this proposal could result in the development of a useful treatment for the control of equine reproduction-producing sterility lasting at least 6 months after each treatment. This would be a tremendous step forward in controlling the wild equine population.

The concepts on which this method are based arose from previous research in reproduction in the dog and in developing anti-cancer drugs targeted to prostate, testicular, ovarian, and breast cancer cells. Hansel, Leuschner and Enright developed a group of compounds based on conjugation of membrane disrupting peptides to either Luteinizing Hormone Releasing Hormone (LHRH) or to a 15-amino acid segment of the beta chain of human chorionic gonadotropin (I3CG). 14 These conjugates are capable of targeting and destroying human prostate, testicular, breast, and ovarian cancer cells and their metastases in the nude mouse cancer model.2 The toxicity of these conjugates to a variety of cancer cells is highly correlated with the number of LHRH and/or LH receptors expressed.3 LHRH- and peG-conjugated ligands to either Phor21 or to the Hecate membrane disrupting peptides were found to inhibit follicular growth and ovulation in female mice and to inhibit spermatogenesis and testosterone production in male mice. These conjugates were found to be active in extremely low doses of 0.008 to 0.02 mg/kg.13 All membrane disrupting peptide conjugates are rapidly metabolized.4

Histological studies revealed that both the LHRH and CG conjugates caused infertility in nude and immunocompetent female mice by destroying both the granulosa and theca cells in primary, secondary, and tertiary follicles but left primordial follicles intact. In the male mice, the membrane disrupting peptides conjugated to either LHRH or CG destroyed differentiating spermatocytes, spermatids, and the testosterone secreting interstitial (Leydig) cells, while spermatogonia remained unaffected. Subsequent studies with normal male pigs, outlined in the Previous Studies section below, showed similar results based on the presence of either LHRH or LH receptors on spermatogenic tissues.

The only significant difference in host cell susceptibility between LHRH and CG conjugates was the selective destruction of gonadotropic cells, which bear the LHRH receptor, in the pituitary gland by the LHRH conjugate. These cells are not destroyed by the CG conjugate as these cells do not express the LH receptor.

Previous Studies

Histological examination of the ovaries of Phor21-CG treated nude mice bearing xenografts of human prostate or breast cancers cells revealed that all theca and granulosa cells in primary, secondary and tertiary follicles were destroyed; only the small primordial follicles survived. In contrast, the ovaries of the control mice contained many developing follicles and many large corpora lutea. Similarly, all of the spermatocytes, spermatids and the testosterone-producing interstitial cells in the testes of the nude mice bearing human prostate cancer cell xenografts and treated with Phor21-CG were destroyed; but the spermatogonia remained intact.

In order to extend these findings to normal mice and to determine if the effects of Phor21-CG treatments are reversible, we tested the effects of the drug in 96 mature female and 96 mature male mice divided into 4 groups of 24 each, injected intravenously once a week for 3 weeks with 0, 0.2, 2.0 or 8 mg/kg of the drug. Three subgroups of 6 animals each were necropsied at 1 week, 1 month, and 3 months after the last Phor21-CG injection; and a fourth group of treated animals was mated 3 months after the last injection with normal, untreated males or females. Data collected during the experiment and after necropsy included: 1) blood concentrations of testosterone (males) and progesterone (females); 2) body and organ weights at necropsy; 3) daily vaginal smears (females); and 4) histopathological examinations of ovaries, testes, pituitaries, adrenals and thyroids of males and females collected at necropsy.

The major results of this experiment may be summarized as follows:

1. Serum progesterone concentrations in female mice declined in the first week after the first injection of Phor21-CG and did not return to normal levels until 12 weeks after the last injection.
2. Serum testosterone concentrations in males were also diminished in the first week after Phor21-CG injection and gradually returned to nearly normal concentrations 12 weeks after the last injection.
3. Combined ovarian and uterine weights in females were significantly reduced, even at 12 weeks after the last injection.
4. Testes weights and body weights (both male and female) were not affected, except for and [increase or decrease] in body weight of females receiving the highest dose.
5. As was the case in nude mice bearing human cancer cell xenografts, all follicle cells, except in the primordial follicle cells, were destroyed in the treated females and all spermatogenic cells except the spermatogonia were destroyed in the males. The interstitial cells were pyknotic or absent. These changes were largely reversed at 3 months after the last injection when fertility in both males and females was restored.

The ability of implants of Phor21-CG to suppress testosterone secretion was tested in post-pubescent male pigs. In one study, five 6-month old boars, each weighing approximately 125 kg, were subcutaneously (SQ) implanted with polycaprolactone implants containing 13-18 mg of Phor 21-CG. Each implant was expected to release 0.06-0.09 mg/kg body weight of peptide over a 20 to 25 day period. Four age and weight-matched boars received SQ implants containing no ligand membrane disrupting peptide. Four of five boars with Phor 21- CG implants demonstrated reduced levels of testosterone (less than 20 ng/ml) for 44 days post-implantation (dpi) with two continuing to have reduced concentrations for 64 dpi. Treatment and control boars were sacrificed 76 to 82 dpi. Control testicles demonstrated active sperm production and dense populations of interstitial cells while Phor 21- CG affected testicles showed disorganized degenerative seminiferous tubules with no mature sperm production. At sacrifice, the interstitial cell populations were recovering but not yet back to those of control animals.

In another study, two boars weighing between 200 to 225 kg received 10 mg Phor 21-CG intravenously (IV) twice in 4 mls of saline at 21-day intervals. Age and weight matched boars receiving saline alone served as controls. All boars were sacrificed seven days following the second injection. Treated boars had depressed testosterone levels prior to the second injection (at 21 days) and at necropsy. An observer blinded to the treatment groups reported that the treated boars were less aggressive than the control animals. Grossly, the Phor 21- CG treated boars exhibited smaller, less turgid testicles than the saline-treated boars. Our studies revealed the complete disruption of the spermatogenic cycle in the seminiferous tubules and near complete loss of interstitial cells in Phor 21- CG treated boars. Only spermatogonia remained; Sertoli cells and developing spermatocytes were absent within the seminiferous tubules.

These preliminary studies in large sexually mature boars utilized low doses of Phor 21-CG that were one half to one twentieth of the lowest dose (0.2 mg/kg) in murine studies[5] yet the duration of testicular disruption in both the mouse and implanted pigs was similar.

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