

***Mycobacterium avium*: Is It an Essential Ingredient for a Single-Injection Immunocontraceptive Vaccine?**

Kelly R. Perry

USDA APHIS WS, National Wildlife Research Center, Olympia Field Station, Olympia, Washington

Lowell A. Miller

USDA APHIS WS, National Wildlife Research Center, Fort Collins, Colorado

Jimmy Taylor

USDA APHIS WS National Wildlife Research Center, Olympia Field Station, Olympia, Washington

ABSTRACT: Greater demand for non-lethal means of alleviating human-wildlife conflict has led to an interest in contraception as a wildlife management tool. The development of a single-injection Gonadotropin-Releasing Hormone (GnRH) contraceptive vaccine by the USDA National Wildlife Research Center reduces logistical limitations and the cost of using a vaccine that requires 2 injections. This study assesses the efficacy of different GnRH-KLH (keyhole limpet hemocyanin) vaccine designs. Forty-two captive female black-tailed deer were divided into 3 groups. A control group was injected with saline solution, and 2 treated groups were given either GonaCon™, a GnRH vaccine paired with AdjuVac™ (an adjuvant containing 175 µg of killed *Mycobacterium avium*), or GnRH vaccine without AdjuVac™ (instead substituting DEAE-dextran/oil (DD) as the adjuvant). Pregnancy rates in deer treated with GonaCon™ were significantly reduced as compared to saline controls ($P = 0.006$). There was no significant difference between GnRH-DD compared to saline ($P = 0.297$). Significant difference was found between GonaCon™ and GnRH-DD ($P = 0.055$). Vaccinated does that remained fertile received booster injections according to treatment group and were administered either GonaCon™ containing 87 µg *M. avium* or GnRH-KLH conjugate bound to DEAE-dextran. Deer that received booster injections regardless of the adjuvant were 100% contracepted for 1 year. Six out of 10 deer that received a prime injection of GonaCon™ remained 100% contracepted for 3 years, suggesting that the killed *M. avium* in the adjuvant is essential for the success of GonaCon™ as a single-injection GnRH vaccine.

KEY WORDS: AdjuVac™, black-tailed deer, contraception, fertility control, GnRH, GonaCon™, immunocontraception, *Mycobacterium avium*, *Odocoileus hemionus*, wildlife population control

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INTRODUCTION

Overabundance of wildlife species often leads to human-wildlife conflict. Black-tailed deer (*Odocoileus hemionus*) are 1 of several deer species whose overabundance results in the potential to cause both biological problems and human-wildlife conflicts (McShea et al. 1997). Lethal removal is a management option frequently used to lower deer density; however, increased land fragmentation and urbanization may prevent the safe use of firearms in many areas. Safety concerns paired with public perception of lethal control as an inhumane approach to population control has led to a growing interest in wildlife contraception and the development of contraceptive vaccines.

Scientists at the USDA National Wildlife Research Center (NWRC) in Fort Collins, Colorado developed a contraceptive vaccine designed to immunize against gonadotropin releasing hormone (GnRH), a hormone essential for mammalian reproduction. GnRH produced in the hypothalamus of the brain is transported to the anterior pituitary where it diffuses from the capillaries and binds to and activates follicle stimulating hormone (FSH) and luteinizing hormone (LH). Activated FSH and LH gonadotrophs trigger the release of stored FSH and LH, which diffuse back into the capillaries where they travel to and activate the reproductive organs. Activated reproductive organs synthesize steroids responsible for sperm production in males and follicular development and ovulation in females, resulting in normal sexual activity (Miller et al.

2000). The vaccine stimulates the production and release of GnRH-specific antibodies that bind to the circulating GnRH and form large immune-complexes. The increase in size prevents normal GnRH diffusion out of the blood at the pituitary capillaries; instead, it exits the pituitary without stimulating the release of FSH and LH, subsequently preventing normal steroid synthesis. Animals remain in a non-reproductive state resulting in temporary infertility (Miller et al. 2000).

Unlike vaccines designed to control diseases, which generally function by priming the immune system to be on alert for infections caused by foreign organisms, the GnRH contraceptive vaccine needs to elicit antibody production against normally occurring hormones. In order to initiate an immune response, synthesized GnRH is conjugated to a foreign carrier protein. The GnRH peptide hormone was made immunogenic by coupling the peptide to keyhole limpet hemocyanin (KLH) (Pierce Endogen, Rockford, IL). KLH is a protein derived from the marine mollusk *Megathura crenulata*. The protein has a high molecular weight (proteins low in molecular weight are generally not immunogenic) and is widely used as a hapten carrier for vaccines and antigens (Harris and Markl 1999, McFadden et al. 2003).

Even though the GnRH-KLH conjugate is immunogenic, in order for the immunocontraceptive vaccine to be effective it needs to continually initiate a high level of anti-GnRH antibody titers. Enough antibodies need to be produced to bind to all the circulating GnRH. In vaccines

for the control of infectious disease, antibody production is accomplished by preparing the immune system to be on the lookout for infection caused by organisms similar to those introduced in the vaccine. Antibody titers against the disease may be absent or low until infection occurs. The infection serves as a booster, stimulating antibody production. Subsequent booster injections are commonly used to provide additional exposure to keep the immune system on the alert. Due to the logistical challenge of administering subsequent injections to free-roaming wildlife, the development of a single-injection vaccine is essential before immunocontraception can become a realistic tool for deer management. The creation of a new adjuvant, AdjuVac™, by NWRC scientists has led to immense progress in the development of a single-injection vaccine with multiple-year efficacy.

An adjuvant is commonly incorporated into vaccine design as a mechanism to improve immune response and to stimulate high levels of antibody production. There are many types of adjuvants, including water-soluble and water-in-oil emulsions. A water-soluble polycationic diethylaminoethylether polymer of dextran (DD) has been shown to be a potent adjuvant for vaccines (Houston et al. 1976, Joo and Emod 1988). In a study of GnRH-ovalbumin conjugate vaccine in white-tailed deer, DD was used as the adjuvant for multiple injections to elicit an immune response against GnRH (Becker et al. 1999); however, multiple-injection vaccines are not practical for most wildlife applications.

AdjuVac™ is an oil-based adjuvant. The GnRH-KLH conjugate, which is water soluble, is mixed as a water-in-oil emulsion with AdjuVac™. Water-in-oil emulsions form a depot of antigen at the site of injection which protects the antigen from rapid degradation, allowing a slow release of the antigen for a prolonged period of time; this is known as the depot effect (Powers et al. 2007). The emulsion protects the antigen from rapid macrophage destruction, providing an antigen source for a period of weeks to months enabling antibody production. It takes approximately 2 to 3 weeks for specific antibodies to be developed against an antigen (Miller et al. 2004). If the antigen is still present when specific antibodies have been developed, they will combine to produce immune-complexes. The immune-complexes will drain into the draining lymph node, providing a protected source of antigen for months, potentially years (Burton et al. 1994).

Water-in-oil emulsions are effective at eliciting an immune response themselves; however, the addition of a bacterial component is often included to increase the immune response. AdjuVac™ contains a small quantity of killed *M. avium* bacterium. The bacteria have a waxy coating that protects them from rapid macrophage destruction, adding to the depot effect and longevity of antigen presence (Freund et al. 1937).

NWRC developed AdjuVac™ by modifying a USDA-licensed vaccine for Johne's disease (Mycopar™, Fort Dodge Animal Health, Fort Dodge, IA). Johne's disease or paratuberculosis is a chronic gastrointestinal infection of ruminants caused by *Mycobacterium avium* ssp. *paratuberculosis* (Paolicchi et al. 2001, Siegmund 1973). Nontuberculosis mycobacteria including *M. avium* are common in the environment (Covert et al. 1999, Falkin-

ham et al. 2001); therefore, it's likely that most domestic and wildlife species have been exposed to the bacteria (Miller et al. 2004). As well as strengthening the initial immune response to the vaccine, by incorporating *M. avium* bacterium, AdjuVac™ effectively elicits a booster effect by tapping into an animal's natural exposure to the bacteria.

Vaccine efficacy depends on a variety of factors including the quantity of antigen necessary to elicit an immune response; too little may simply be tolerated without triggering an immune response, while too much may lead to health problems. Freund's Complete Adjuvant (FCA), a water-in-oil emulsion adjuvant with a bacterial component (*M. tuberculosis* or *M. butyricum*) is extremely effective; however, hazards associated with its use (Claassen et al. 1992, Powers et al. 2007), have led to increased restrictions in its use (Claassen and Boersma 1992). The amount and type of *mycobacterium* incorporated into the adjuvant may affect the likelihood and severity of adverse effects (Powers et al. 2007). AdjuVac™ contains a different *mycobacterium* and smaller quantities than FCA, which may result in it being a safer adjuvant (Powers et al. 2007). Assessment of the different components involved in vaccine design is critical in the development of an effective, single-injection immunocontraceptive vaccine that has a low risk of adverse effects.

A need to reduce reproductive rates of black-tailed deer held at the NWRC Olympia Field Station (OFS) resulted in the implementation of an experiment designed to help reach this management objective and at the same time enabled collection of data on black-tailed deer responses to immunocontraceptive vaccines. The study was designed to assess the contraceptive efficacy of the GnRH vaccine with AdjuVac™ (GonaCon™), compared to the GnRH conjugate vaccine with DEAE-dextran (GnRH-DD) as the adjuvant.

VACCINES

The GnRH used in this study was synthesized at Macromolecular Resources, Colorado State University (Fort Collins, CO). The second treatment vaccine was paired with the adjuvant diethylaminoethyl-dextran (DD) (Sigma-Aldrich, St. Louis, MO), a polycationic derivative of dextran. The aqueous-based GnRH-KLH conjugate bound to DEAE-dextran was combined in a 1:1 ratio by volume with AdjuVac™ diluent.

CAPTIVE BLACK-TAILED DEER STUDY

The study was conducted at the OFS of the NWRC in Washington State. Thirty-nine adult female black-tailed deer reared at the OFS, individually identified by ear tags, were used for this study. The deer were stratified by age and then randomly assigned to one of 3 treatment groups such that all ages were equally represented across groups. Age ranged from 1 to 6 years with an average age of 3 years in each treatment group. The treatments included GonaCon™ vaccine (1,000 µg GnRH-KLH in AdjuVac™ adjuvant, $n = 13$), GnRH-DD vaccine (1,000 µg GnRH-KLH in DD/oil adjuvant, $n = 13$), and a saline control group ($n = 13$). Treatments were administered on 22-23 September 2004. Deer handling facilities at the OFS consist of a series of holding boxes that lead to a Deerhan-

dlar™ squeeze chute (Delclayna Whitetail and Bison Co. Ltd., St. Albert, Alberta, Canada). Deer were restrained and a 10-ml blood sample was collected. Serum was then separated for analysis of anti-GnRH and anti-Johne's antibody titers (*M. avium*). Does were injected intramuscularly with their assigned treatments. Does were returned to their normal pastures and isolated from bucks for approximately 10 weeks to enable the vaccine to stimulate sufficient antibody titers. Treated does were penned with bucks from 15 November 2004 through 20 March 2005 to allow breeding. Blood collections were repeated 17 February 2005 for assessment of anti-GnRH and anti-Johne's antibody titers post vaccination.

Deer were monitored for pregnancy and fawn production was recorded. Fawns were individually identified with ear tags and associated with does through behavioral and physical observations. Anti-GnRH and anti-Johne's antibody titers were analyzed by treatment using an ANOVA with a post-hoc Tukey t-test (SAS Inc. 9.1, Cary, NC). Pregnancy rates by treatment were compared using Pearson's Chi-Square test (SPSS Inc. 14.0, Chicago, IL). Fisher's Exact Test was used to further compare the efficacy of the treatments.

In August 2005, a second set of treatments was administered to determine the effectiveness of the GonaCon™ vaccine with lower concentrations of *M. avium*. The amount of *M. avium* in traditional GonaCon™ is 175 µg. Fawn-producing does in the GonaCon™ treatment group received a GonaCon™ booster vaccine containing 87 µg of killed *M. avium* bacterium ($n = 4$). Contracepted does in the GonaCon™ treatment group were not given any additional vaccinations ($n = 6$). All of the remaining subjects in the GnRH-DD group received a booster injection of the GnRH-KLH conjugate bound to DEAE-dextran ($n = 10$). Deer from the saline group plus 4 new subjects were given a prime vaccine of GonaCon™ containing either 44 µg ($n = 5$) or 175 µg ($n = 8$) of killed *M. avium*

bacterium. Deer were monitored for pregnancy and fawns in 2006 and 2007.

Serum Analyses

Serum was assayed for GnRH antibodies by enzyme-linked immunoabsorbant assay (ELISA). Deer serum was tested at serial dilutions to endpoint titers. Two negative controls were run on each plate; one negative control was buffer without deer serum and the other was pre-vaccination deer serum. High-titer deer serum was used as the positive control.

RESULTS

Pregnancy rates in deer were significantly different based on treatment ($X^2 = 9.389$; $df = 2$; $P = 0.009$). Pregnancy rates in deer treated with GonaCon™ were significantly reduced as compared to control ($P = 0.006$), but there was no significant difference in pregnancy rates between GnRH-DD compared to control ($P = 0.297$). A significant difference in pregnancy rates was found between GonaCon™ and GnRH-DD ($P = 0.055$).

Anti-Johne's anti-bodies were higher in deer vaccinated with GonaCon™ compared to deer vaccinated with GnRH-DD ($P < 0.05$) (Table 1). Mean anti-Johne's antibodies were approximately the same pre- and post-treatment for both the control and GnRH-DD treated deer. Mean anti-Johne's antibodies increase in the GonaCon™ group post vaccination. None of the deer had detectable anti-GnRH antibody titers prior to treatment, and control deer had no detectable anti-GnRH titers post-treatment. Post-treatment anti-GnRH antibody titers differed significantly between treatment groups ($df = 2$; $P = 0.022$). Anti-GnRH antibody titers were higher in deer treated with GonaCon™ compared to both control deer and deer treated with GnRH-DD ($P < 0.05$).

Six of the deer from the 2004 GonaCon™ treatment group that only received a prime injection remained 100%

Table 1. GnRH and Johne's antibodies are compared between the control and two treatment groups. Also, the percent pregnancy is compared for the first year between groups.

Treatment	GnRH Antibody Titers		Johne's Antibody Titers (<i>M. avium</i>)		Pregnancy
	9/22/04	2/17/05	9/22/04	2/17/05	2005
Saline	0 ± 0	0 ± 0	0.27 ± 0.11	0.25 ± 0.10	92%
GnRH-DD	0 ± 0	7.54 ± 4.87	0.13 ± 0.08	0.11 ± 0.06	77%
GonaCon-AdjuVac™	0 ± 0	32.38 ± 13.33	0.20 ± 0.13	0.44 ± 0.14	38%

Table 2. Percent pregnancy is compared between groups for the 3 years of the study. In 2005, a boost was given to animals in all treatments, except the 6 does from the GonaCon group that remained infertile throughout the 3-year study.

2004 Treatment	# of Does	Pregnancy 2005	# of Does	2005 Treatment	Pregnancy 2006*	# of Does	Pregnancy 2007
GonaCon™ 175 µg <i>M. avium</i>	13	38%	4	booster 87 µg <i>M. avium</i>	0%	3	33%
			6	no treatment (contracepted deer from 2004)	0%	5	0%
GnRH-DD	13	77%	10	booster GnRH-DD	0%	9	33%
Saline	13	92%	5	prime 44 µg <i>M. avium</i>	20%	5	60%
			8	prime 175 µg <i>M. avium</i>	38%	8	50%

*0% pregnancy is equal to 100% contraception in the treated groups

contracepted for 3 years (Table 2), suggesting that the killed *M. avium* in the adjuvant is essential for the success of GonaCon™ as a single-injection GnRH vaccine. Deer that received booster injections regardless of the adjuvant (GonaCon with 87 µg *M. avium*, GnRH-DD) were 100% contracepted for 1 year and 67% contracepted for the second year post booster injection. Subjects from the 2005 treatment group received a prime vaccine of GonaCon™ containing either 44 µg (*n* = 5) or 175 µg (*n* = 8) of killed *M. avium* bacterium were 80% and 62% contracepted for 1 year and 40% and 50%, respectively, for the second year post-vaccination. These results suggest that small quantities of *M. avium* are critical for vaccine efficacy; however, due to the small sample size, further testing is needed to determine the quantity of *M. avium* necessary for single-shot vaccine efficacy.

DISCUSSION

This study demonstrated that GonaCon™ was effective in reducing pregnancy rates of captive black-tailed does for at least 3 years. Results suggest that the killed *M. avium* in the AdjuVac™ adjuvant is essential for the success of the single-injection GnRH vaccine GonaCon™. The efficacy of *M. avium* may be attributed to the natural exposure of deer to *M. avium* bacteria in their environment; 34 of the deer in this study had antibodies against *M. avium* (Johne's disease) prior to vaccination. The GnRH AdjuVac™ treatment group was the only group that had an increase in mean anti-*M. avium* antibody titers post vaccination as well as the highest amount of anti-GnRH antibodies. The continued exposure to *M. avium* in the environment may help initiate the continued immune response, thus high antibody titer levels necessary for multi-year contraception without the need of a booster injection. However, the immune mechanism that allowed the 6 deer to remain contracepted for 3 years without a boost is unknown at this time.

The development of a single-injection vaccine will increase the practicality and lower the cost of using immunocontraception in the control of overabundant deer. Contraceptive vaccines are not a panacea, rather they may be a beneficial tool when incorporated into integrated pest management plans.

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