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TWENTY YEARS OF IMMUNOCONTRACEPTIVE RESEARCH: LESSONS LEARNED

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Abstract: The National Wildlife Research Center (NWRC) began immunocontraception vaccine research by testing porcine zona pellucida (PZP) on white-tailed deer (*Odocoileus virginianus*). Early PZP research demonstrated that PZP induced infertility; however, increased length of the rut was observed in PZP-treated deer. An alternative vaccine using a keyhole limpet hemocyanin-gonadotropin-releasing hormone (KLH-GnRH) conjugate formulated with modified Freund's adjuvant was developed at NWRC. Suppression of GnRH has reduced reproduction in both sexes but is most effective in females. This vaccine was effective in preventing contraception in female deer for several years after a prime and boost. Due to adverse side effects of Freund's adjuvant, NWRC developed a new adjuvant called AdjuVac, a mineral oil/surfactant adjuvant with the addition of *Mycobacterium avium* as an immunostimulant. The price of KLH prompted a search for a more economical hemocyanin carrier protein for the GnRH peptide. Blue protein, derived from the mollusk *Concholepas concholepas*, proved to be a successful option. Formulation improvements resulted in a vaccine that can be effective as a single injection for multiple years, now called GonaCon. GonaCon is registered with the Environmental Protection Agency (EPA) for use in white-tailed deer in urban/suburban areas and for wild horses (*Equus caballus*) and burros (*Equus asinus*). Future GonaCon applications may include reducing reproduction to manage populations of other wildlife species, such as prairie dogs (*Cynomys ludovicianus*) in urban areas and suppressing reproduction to reduce the spread of venereal diseases such as brucellosis. Research is being conducted to develop a GnRH vaccine used in combination with the rabies vaccine to control population growth in free-roaming dogs, with the secondary effect of managing the spread of rabies. The EPA would regulate all these uses. Research is also ongoing on a GnRH vaccine to delay the onset of adrenocortical disease in pet ferrets (*Mustela putorius*), a use regulated by the United States Department of Agriculture.

Key words: AdjuVac, GonaCon, immunocontraceptive vaccine, KLH, *Mycobacterium avium*.

INTRODUCTION

In 1992, the United States Department of Agriculture National Wildlife Research Center (NWRC) began a project to develop reproductive control methods as an additional nonlethal tool to manage overabundant wildlife. The early focus of the project was on white-tailed deer (*Odocoileus virginianus*) because stakeholders identified overabundant deer populations in urban/suburban areas as a concern because of increases in deer-motor vehicle collisions, property damage, and habitat destruction. The project began by studying porcine zona pellucida (PZP) immunocontraception technology for management of white-tailed deer.[23,47,48] The project's focus later shifted to development of vaccines using gonadotropin-releasing hormone (GnRH) as the antigen for several reasons: first, the PZP vaccines are only effective in females and resulted in prolonged periods of estrus cyclicity because normal reproductive hormones were maintained; second, the project's focus broadened beyond white-tailed deer, and a broadly effective vaccine in most mammals was wanted that would affect both sexes; and third, other researchers were developing PZP vaccines, so duplicate research efforts needed to be minimized. Initial studies were conducted with various formulations of GnRH vaccines. Eventually, the vaccine GonaCon was developed at NWRC, which suppresses reproductive hormone production, estrus, and spermatogenesis in mammals, often after a single injection. For this review, GonaCon is a GnRH-based immunocontraceptive vaccine comprised of numerous GnRH peptide molecules coupled to a mollusk carrier protein (either keyhole limpet hemocyanin [KLH] or blue protein [*Concholepus concholepus*]) mixed with a mineral oil-based adjuvant containing *Mycobacterium avium* (AdjuVac) and made into an emulsion (Table 1). Generally, efficacy for longer than 1 yr is achieved from a single injection, and contraception may be extended by administering a boost vaccination.[48,50] GonaCon proved efficacious in deer, reducing

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Table 1. Formulations for GnRH vaccines tested by the National Wildlife Research Center.

Name of formulation[a]	Species tested	Mollusk protein[b]	Adjuvant used[c]	Amount of *M. avium*[d]	Citation
GnRH vaccine	White-tailed deer	KLH	FCA for prime, FIA for boost	NA	23, 29, 42, 48
GnRH vaccine	White-tailed deer fawns	KLH	FCA, FIA boost	NA	43
GnRH vaccine	Norway rats	KLH	FA prime; FIA boost	NA	44
GnRH vaccine	New Zealand white rabbits	KLH	FCA; FIA; AdjuVac	Standard	57
GonaCon	White-tailed deer	KLH and blue	AdjuVac	Standard	42
GonaCon	Black-tailed deer (*Odocoileus hemionus*)	KLH	AdjuVac	Standard, one-half standard, one-fourth standard	55
GonaCon	Elk	KLH	AdjuVac	Standard	22
GonaCon	Elk	Blue	AdjuVac	Standard	56
GonaCon	Bison	KLH	AdjuVac	Standard	50
GonaCon	Domestic swine	KLH	AdjuVac	Standard	51
GonaCon	Feral swine	KLH	AdjuVac	Standard	26, 27
GonaCon	Wild boar	KLH	AdjuVac	Standard	37
GonaCon	California ground squirrel	KLH	AdjuVac	Standard	53
GonaCon	Eastern gray squirrel	Blue	AdjuVac	Standard	54
GonaCon	Black-tailed prairie dog	Blue	AdjuVac	Standard	66
GonaCon	Domestic cats	KLH	AdjuVac	Standard	32, 34
GonaCon	Brushtail possum	KLH	AdjuVac	Standard	8
GonaCon	Tammar wallaby; Gray kangaroo	Blue	AdjuVac	Standard	60
GonaCon	Domestic ferret	KLH and blue	AdjuVac	Standard	40
GonaCon-Cervid (EPA registration)	White-tailed deer	KLH	AdjuVac	Standard	13, 14, 21
GonaCon-Equine (EPA registration)	Wild horses	KLH	AdjuVac	Standard	24, 25, 28
GonaCon-Equine (EPA registration)	Wild horses	Blue	AdjuVac	Standard	18
GonaCon	Dogs	KLH	AdjuVac	Standard	19
GonaCon	Dogs	KLH	AdjuVac	One-half standard	2
GonaCon-Canine (Proposed EPA registration)	Dogs	Blue	AdjuVac	One-fourth standard	63

[a] GonaCon is defined as a GnRH immunocontraceptive vaccine comprised of numerous GnRH protein molecules coupled to a mollusk carrier protein (either keyhole limpet hemocyanin [KLH] or blue protein) mixed with a mineral oil-based adjuvant (AdjuVac) and made into a stiff emulsion.

[b] The EPA registered formulations for GonaCon can contain either keyhole limpet hemocyanin (KLH), or blue protein (*Concholepas concholepas*).

[c] GonaCon is formulated with the adjuvant Adjuvac, a dilution of Mycopar, which contains *Mycobacterium avium*. FCA, Freund's Complete; FIA, Freund's Incomplete.

[d] The standard amount of *M. avium* (approximately 0.17 mg/ml) is the concentration in AdjuVac for the Environmental Protection Agency (EPA) registered products. NA, not applicable.

reproductive behaviors in females and reducing the length of the rut;[13,23] it was also found to induce infertility in many different species of mammals.[9] NWRC has subsequently improved the manufacturing process for GonaCon to include aseptic manufacturing in a clean room, as well as scaling up production to allow GonaCon to be made in larger batches. An adjuvant (AdjuVac) was developed by the NWRC to increase the effectiveness of both PZP and GnRH vaccines.

This manuscript will briefly describe much of the research conducted by the NWRC during the last 20 yr, the vaccines and adjuvant that have been developed, the changes that have been made to improve those products, the lessons NWRC has learned, and what still needs to be determined after 20 yr of research on immunocontraception in wildlife.

VACCINE DESIGN FOR DIFFERENT REPRODUCTIVE SYSTEM TARGETS

Immunocontraceptive vaccines use the animal's immune system to produce antibodies against gamete proteins, reproductive hormones, and other proteins essential for reproduction.[30] The antibodies interfere with the biologic activity of the reproductive proteins,[61] and depending on the antigen and formulation, the vaccines can be effective for 1–4 yr or longer with single or multiple injections.[47,62] Two antigens, PZP and GnRH, have been used to develop immunocontraceptive vaccines for wildlife. The NWRC has conducted research on both PZP and GnRH vaccines. The antigen used, including its mode of action on the reproductive system and the design of the vaccines, is vital in determining vaccine efficacy in different mammalian species.

PZP immunocontraceptive vaccines

The NWRC began its contraceptive research program in 1991 by studying PZP, a combination of three zona pellucida (ZP) proteins extracted from pig ovaries, because PZP had previously been shown to be effective as an antigen in an immunocontraceptive vaccine used for horses (*Equus caballus*).[35] In the authors' early work, it was effective in coyotes (*Canis latrans*) as well.[38] The PZP vaccine targets the ZP, a glycoprotein layer located on the outer surface of the egg. Antibodies to ZP result in infertility either by blocking sperm from penetrating the ZP layer or by interfering with egg maturation within the follicle.[6]

Extracted PZP proteins were initially provided to the NWRC by Dr. Bonnie Dunbar (Baylor College of Medicine, Houston, Texas, 77030, USA) and Dr. Irwin Liu (University of California-Davis, Davis, California 95616, USA) and were produced by the method of Dr. Dunbar.[7] For later NWRC studies, native PZP and SpayVac (PZP encapsulated in liposomes) were produced and provided by Dr. Robert Brown (ImmunoVaccine Technologies [IVT], Nova Scotia, B3H OA8 Canada).[3]

During early research with PZP prepared by the Dunbar method, 11 white-tailed deer does at Pennsylvania State University (University Park, Pennsylvania 16802, USA) received a prime vaccination in July to August and two boost vaccinations in September and October; in November, the does were paddocked with bucks. Extensive observations of breeding activity by a team of Penn State students were made comparing the breeding activity of female deer vaccinated with the vaccine. This PZP preparation was quite effective in preventing pregnancy when deer received both prime and boost doses, with 89% reduction in fawning over a 4-yr period and 76% over 6 yr.[47]

After demonstrating that PZP vaccines could be effective when given as a prime and a boost, NWRC researchers attempted development of a vaccine that could induce a long-lasting immune response after a single injection. A study was subsequently conducted comparing the effectiveness of six different single injection vaccine formulations,[41] containing different PZP preparations (Dunbar preparation, IVT preparation without liposomes, and SpayVac). The study demonstrated that the different production methods for PZP affected the duration of effect of the resulting vaccines. The PZP isolated by IVT produced a longer-lasting response as compared witj the Dunbar PZP isolation method. The IVT PZP with liposomes (SpayVac) and without liposomes were the most efficacious formulations, with 8 of the 10 does in each group contracepted for 5 to 7 yr after a single injection.[41] The normal fertility level for this herd was 1.7 fawns/doe per year.[42] The deer study also compared different adjuvants used with the SpayVac PZP: SpayVac prepared in AdjuVac; lypophilized SpayVac suspended in AdjuVac; and SpayVac mixed with alum as the adjuvant. The water-in-oil emulsion produced with AdjuVac provided a longer-lasting response than the alum or suspension.[41] NWRC researchers were encouraged that vaccines formulated

with AdjuVac provided the long-lasting efficacy with a single injection that would be required for most uses in wildlife.

Because the process of isolating PZP from pig ovaries obtained from pig slaughterhouse facilities is costly and time consuming, an alternative source of PZP was sought. In one study,[45] different recombinant rabbit ZP proteins produced in *Escherichia coli* were studied. The proteins RC55, RC75a, and RC75b all showed cross reactivity with native PZP. Deer vaccinated with these recombinant proteins were compared with deer vaccinated with native PZP. The native PZP was much more effective in contracepting deer than the rabbit recombinant proteins. In another study,[49] the NWRC used selected PZP-derived peptides (generated by Chiron Mimotope Systems, San Diego, 92121, California) identified from published sequence information (National Institutes of Health gene bank, www.ncbi.nlm.nih.gov/genbank) to identify which peptide or peptide groups were responsible for the contraceptive effect. The peptides were screened using serum from a PZP-treated doe, and a ZP1 mimitrope (pins 10–16) demonstrated the greatest response. When this ZP1 peptide was used to vaccinate a group of nine deer, some efficacy was observed, but there was also large variation in the immune response between deer. The deer with the highest antibody titer to this ZP1 peptide exhibited three estrous cycles and had no fawns; however, the average fawning rate in this group was 0.89 fawns/doe, which was indicative of a partial contraceptive effect. It was concluded that the native PZP, which apparently maintains the natural folding of the glycosylated protein, was more consistent in inducing infertility than this or any of the other peptides tested.[49]

By effectively blocking sperm from penetrating the egg without reducing circulating hormone levels, PZP vaccines caused repeated cycling during the rut season in deer at Penn State University,[23] extending the breeding season for the PZP-treated deer for up to seven estrus cycles ($\bar{x} = 2.4$ cycles), with a mean of 98 days and a few deer cycling up to 150 days.[47] This increased length of the breeding season has the potential for increasing car-deer collisions because of increased deer movement. It also causes increased energy expenditures by rutting deer, and if antibody titers drop below a critical threshold late in the breeding season, late season fawns can be produced that may not survive the northern winter climates. Moreover, other researchers were developing PZP vaccines,[30] so duplication of research efforts needed to be minimized. For these reasons, the NWRC discontinued testing of PZP in deer and focused research on developing an alternative contraceptive vaccine for use in ungulates.

GnRH immunocontraceptive vaccines

The NWRC researchers desired a vaccine that would eliminate most reproductive behaviors. GnRH is a key reproductive hormone that controls steroidogenesis and gametogenesis by stimulating the release of gonadotropins from the pituitary gland, triggering the cascade of reproductive hormones that lead to sperm production and ovulation. The NWRC began development of GnRH vaccines in the early 1990s, and the first GnRH vaccine was tested at Penn State University beginning in 1994[23,46] (Table 1). This GnRH vaccine was given to females ($n = 8$) and males ($n = 4$) as a prime injection followed by a boost 1 mo later. Extensive observations of breeding activity were made. Fawning rates in the study were 0.21 fawns/doe per year over 4 yr in the treated does compared with 1.8 fawns/doe per year in control does. The contraceptive activity was correlated with the antibody titer for the vaccine, with fertility returning as the antibody titers dropped. GnRH-treated does showed no breeding behavior, and bucks had no interest in them; the reduction in progesterone shown in the GnRH-treated does suggested that they were not cycling. GnRH immunized bucks had a significant reduction in testosterone and had no interest in sexual activity when paired with control females. Depending on the immunization schedule, antlers in GnRH-treated bucks either dropped early in the fall or remained in velvet.

After years of refinement, a new vaccine (GonaCon Immunocontraceptive Vaccine, NWRC, Fort Collins, Colorado, 80521, USA) was developed by the NWRC that has been shown to suppress reproduction in treated animals of both sexes, can be administered as a single injection,[52] is effective for at least a year in most mammalian species,[9] and is often effective for multiple years.[9] Like earlier GnRH vaccines, GonaCon stimulates the production of antibodies that neutralize GnRH, suppressing reproductive hormone production (i.e., follicle-stimulating hormone [FSH] and luteinizing hormone [LH] from the pituitary gland and estradiol, progesterone, and testosterone from the gonads). The design of GonaCon is the key to its long-term efficacy with a single injection; the

characteristics of the design that allow it to be effective without a boost injection will be discussed in further detail.

VARIATION IN VACCINE EFFICACY AMONG INDIVIDUAL ANIMALS

Users of a contraceptive vaccine need to be aware that, unlike surgical sterilization, which is 100% efficacious, the efficacy of immunocontraceptive vaccines is subject to individual variation, so it is difficult to predict the percent and duration of induced infertility. A certain percentage of vaccinated animals will always be nonresponders or have a reduced immune response to a vaccine.[59] This is true for both disease vaccines and PZP and GnRH immunocontraceptive vaccines. For human or companion animal vaccines, it is possible to give an injection and a follow-up boost that increases the effectiveness of the vaccine and may provide almost 100% efficacy. For wildlife or feral animals, a boost injection is less desirable because the animal must be relocated, it is difficult to individually mark animals, and it is difficult and time consuming to handle an animal more than once. A single injection, long-lasting contraceptive vaccine like GonaCon or SpayVac is a more attractive wildlife management tool because animals would need to be handled or darted less frequently. However, additional studies and/or population models[20,67] will be required to provide managers with recommendations for implementing contraceptive programs as management tools to reduce or maintain wildlife population levels.

Contraceptive vaccine users also need to know that, while GonaCon is effective in both males and females, it has been demonstrated to be effective for a longer period of time in females than in males. In a GnRH vaccine study at Penn State University, male deer ($n = 5$) given a single injection showed reduced testicular size and plasma testosterone for at least three breeding seasons.[29,46] Males given two immunizations tended to remain infertile for an additional 2–3 yr. In contrast, six of nine female deer were contracepted for 4 to 6 yr after a single injection.[41] Similar differences between longevity of vaccine effectiveness in males and females have been demonstrated with cats (*Felis catus*). When a single injection of GonaCon was given to 15 female cats, 93% of vaccinated cats remained infertile for the first year, with 73, 53, 40, and 27% infertile for 2, 3, 4, and 5 yr, respectively. The median duration of contraception was 39.7 mo based on suppression of hormone levels.[32] In a similar study with males given a single injection of GonaCon, 9 of 12 cats responded with high antibody titers and a drop in testosterone to a nondetectable range; however, three treated cats failed to produce a high GnRH titer and showed no suppression of testosterone.[34] The median duration of effect for the male cats that responded to the vaccine was 14 mo, with one treated cat having undetectable testosterone at 34 mo.

As indicated previously, GonaCon-treated white-tailed deer showed a reduced number of estrus cycles or did not cycle at all, an effect probably due to the lack of estrogen. This reduced breeding behavior associated with infertility was typically seen during the first few years following vaccination. Although the single injection of GonaCon induced infertility for multiple years, an occasional return to breeding behavior 4–5 yr after vaccination, prior to the return of fertility (Miller, unpubl. data),[42] was observed. This return of breeding behavior prior to the return of fertility has also been observed in wild horses (Gray, unpubl. data)[18] and in wild boar (*Sus scrofa*).[37] It is speculated that the reason for the return of breeding behavior prior to the return of fertility could be that GonaCon controls LH levels more completely than it controls levels of FSH. As the level of GnRH antibodies drops in immunized animals over several years, sufficient gonadotropins are released to allow estrogen to reach levels that can induce signs of estrus, but GnRH is still sufficiently inhibited to block the LH surge and ovulation. As the GnRH antibody titer drops further, more LH is released, ovulation resumes and the animal can become pregnant. A vaccine boost will enhance the longevity of the vaccine's efficacy in both males and females. Male white-tailed deer were given a boost of GonaCon that lengthened the time that they remained infertile by several years.[29] In a study with female bison (*Bison bison*) at the Northwest Trek Zoo in 2001 (Eatonville,Washington, 98328, USA), a prime dose was effective in contracepting three of five bison; however, a boost given 1 yr later rendered all bison infertile the second year, and they remained infertile for at least 3 yr.[58] Untreated bison in the herd calved normally.

The authors' research has also demonstrated the importance of timing when injecting the vaccine. The vaccine is most effective if given at least a month or more before the onset of reproductive activity. This allows time for an adequate titer level to develop so contraception can occur.[42]

UNDERSTANDING THE SINGLE INJECTION PRINCIPLE

There are very few vaccines on the market that are effective with a single injection, so the statement that GonaCon is effective in contracepting animals with a single injection is often received with skepticism, especially from knowledgeable immunologists. GonaCon is effective as a single injection due to three primary factors: 1) the GnRH antigen is coupled to a large foreign molecule in a systematic manner; 2) the vaccine antigen is made into a stable emulsion; and 3) *M. avium* is included in the adjuvant.

GnRH is a small peptide hormone that is a weak antigen due to its low molecular weight and its being a "self" hormone. However, due to its small size, GnRH can easily be made synthetically. In the GonaCon vaccine, GnRH is made immunogenic by ensuring that each synthetic GnRH peptide molecule is conjugated in a very systematic and predictable manner to a large, nonself, highly immunogenic hemocyanin protein harvested from marine mollusks;[51,52] thus producing a GnRH-mollusk protein conjugate. Many pathogens, including viruses and bacteria, exhibit rigid, highly organized, and highly repetitive surface protein epitopes. The GonaCon design provides a consistent alignment of up to 300 GnRH peptide molecules on the surface of the large mollusk protein, mimicking the repetitive nature of pathogen epitopes, an important aspect of the GnRH-hemocyanin conjugate design. The original mollusk protein used was KLH. However, the high price of KLH (now used in cancer therapy vaccines) prompted a search for a more economical hemocyanin carrier protein for the GnRH peptide. Blue protein, derived from the mollusk *Concholepas concholepas,* proved to be a successful option. The single injection blue protein formulation has been shown to provide a longer-lasting effect compared with the KLH preparation in white-tailed deer.[39,42]

This water-soluble GnRH/mollusk conjugate is then made into a water-in-oil emulsion with the adjuvant AdjuVac. The mineral oil-based emulsion provides a depot effect for the vaccine and protects the antigen from rapid destruction by the macrophages of the target animals' immune system.[39] This provides for a strong prolonged immune response because of the slow release rate of the GnRH conjugate antigen at the injection site. Remnants of vaccine have been observed at the injection site during necropsy up to 2 yr after vaccination (unpubl. obs.). A water-in-oil emulsion appears to be the only formulation that provides a long-term contraceptive response in both PZP and GnRH vaccines with a single injection.

In all of the successful immunocontraceptive vaccine studies, adjuvants have been used as part of the immunocontraceptive vaccine to enhance the immune response of the host animal to the PZP or GnRH antigen. Adjuvants for contraceptive vaccines are critical in providing efficacy, but unfortunately, the immunostimulatory properties of some of the most effective adjuvants can result in localized inflammation and tissue destruction.[1] In initial research on white-tailed deer, the PZP used by the NWRC was formulated as an emulsion with Freund's Complete Adjuvant for the first injection and Freund's Incomplete Adjuvant for subsequent boosts. During discussions with the Food and Drug Administration (FDA) regarding the potential for gaining regulatory approval of immunocontraceptives, the FDA stated their opposition to the use of any vaccine containing Freund's adjuvant because of its known inflammatory effects. The NWRC began work on a safer adjuvant in 1998, leading to development of a new oil-based adjuvant called AdjuVac, which for currently registered uses contains $<$200 μg of killed *M. avium* per 1-ml dose. Inflammatory reactions caused by AdjuVac are less severe than those caused by Freund's complete adjuvant.[2,57,] AdjuVac has been used as the adjuvant for both PZP and GnRH vaccines by the NWRC since 1998.

In addition to the stable emulsion, the long-lasting contraceptive effect of GonaCon is dependent on the presence of *M. avium* in the adjuvant. In early NWRC studies with PZP immunocontraceptive vaccines in deer using Freund's adjuvant, it was necessary to boost with incomplete Freund's at least once, and sometimes several times, to maintain high antibody titers. When it was switched to the new adjuvant AdjuVac, which contains killed *M. avium*, it was found that in most animals (e.g., deer,[42] horses,[24] and prairie dogs [*Cynomys ludovicianus*][66]) a boost was not needed to achieve a long-lasting immune response. *Mycobacterium avium* has been shown to be ubiquitous in nature,[10] having been recovered from almost every environmental compartment that has been investigated, including fresh water, brackish water, biofilms, aerosols, soils, food plants, plant products, and fish. Furthermore, isolation from a hospital water system over a period of 18 mo demonstrated that *M. avium* is commonly found in drinking-water because of its high resistance to ozone and chlorine-based

disinfectants.[31] The authors hypothesize that due to the ubiquitous nature of *M. avium,* it is a key factor in the success of GonaCon.[39,42,55] Most animals will have previously experienced and produced an immune response to *M. avium*; therefore, the immune system is already primed, leading to a more immediate response to the vaccine than would normally be expected after only a single dose.

Current research from the authors' laboratory has shown that a single injection of Gonacon can provide a multiyear contraceptive effect and has also shown that prolonged infertility with GonaCon is directly related to high serum concentration of GnRH antibodies.[42] Burton et al.[4] writes, "To be effective in producing long-lasting antibodies, antigen administered in a single dose must be retained in the body long enough to produce specific antibodies that will bind with the antigen to form immune complexes (ICs)." Fukanoki et al.[11,12] demonstrated in chickens that the slow release of antigen from oil-based emulsions was positively correlated to immune response. A slow antigen release allows ICs to form, creating a greater immune response. These ICs can then bind to follicular dendritic cells (FDCs) that protect the antigen from macrophage and liver degradation. ICs may begin to form within 7–14 days after a primary injection or within minutes after a booster. The FDCs can provide the continued presence of antigen for months to years.[36] Antigen release from the FDCs relates to the level of the antigen-specific antibody. When specific antibody levels drop, the FDCs will release the specific antigen, boosting the circulating antibody.[41] It is believed that the carefully prepared emulsion used in GonaCon serves as a depot, allowing retention and slow release of the target antigen (i.e., GnRH).

USE OF GONACON FOR REPRODUCTIVE, BEHAVIOR, AND DISEASE MANAGEMENT

GonaCon was initially developed as a management tool for urban/suburban populations of the ever-expanding white-tail deer populations that can be used when other management tools, such as hunting, are impractical or not legal. However, it soon became apparent that a GnRH-based contraceptive could apply to other species as well. Because GnRH is common to all mammals, the GonaCon immunocontraceptive vaccine has induced contraception in many overabundant mammalian species, including white-tailed deer,[47] elk (*Cervus elaphus*),[22] wild horses,[24,25,18] bison,[50] California ground squirrels (*Spermophilus beecheyi*),[53] prairie dogs,[66], gray squirrels (*Sciurus carolinensis*),[54,65] captive Norway rats (*Rattus norvegicus*),[44] domestic and feral swine and wild boar,[26,27,51,37] brushtail possums (*Trichosurus vulpecula*),[8] and tammar wallabies (*Macropus eugenii*).[59] In the United States, all of these uses in wildlife will be regulated by the Environmental Protection Agency.

GonaCon is also being tested as a technique to assist in control of feral or wild dogs and in management of rabies. Rabies in domestic dogs (*Canis familiaris*) has been eliminated in most of the developed world through extensive vaccination, and now rabies only persists in wildlife in these areas.[64] However, in the developing world and in poverty-stricken areas, domestic dogs remain the principle vectors of rabies, with dog bites contributing to more than 95% of human rabies cases.[5] Effective rabies control relies on a combination of large-scale vaccination as well as effective dog population control strategies. Most population control programs have moved away from culling of animals toward surgical sterilization, which is expensive, labor intensive, and does not always reach enough of the dog population to be effective. GonaCon is being investigated as a tool to provide contraception of dogs through a single injection that can be administered in conjunction with a rabies vaccine during annual vaccination campaigns. Simultaneous injection of GonaCon with a canine rabies vaccine in dogs did not affect the development of rabies antibodies.[2] Further research is being conducted to determine the effectiveness of combined contraceptive and rabies vaccine programs as a rabies control tool in field situations.

GonaCon has also been tested in zoo animals, livestock, and companion animals for situations in which animals are not intended to be bred. It has induced contraception in domestic cats,[32,33] and domestic swine.[26,27] GonaCon has also been proposed as a technique for eliminating unwanted behaviors associated with reproduction in companion or work animals. However, because the current GonaCon formulation is designed to last for several years, which may not be desirable in certain situations, the formulation would need to be redesigned to last only a few months to a year as needed. Regardless, uses of GonaCon in domestic or zoo animals would be regulated by the FDA's Center for Veterinary Medicine; these uses will not be pursued by the NWRC but could be pursued by collaborators.

Contraception with GonaCon offers potential as a disease management tool for certain diseases,

most notably, venereally transmitted diseases or diseases transmitted at parturition, such as brucellosis. Swine brucellosis is transmitted through the venereal route as well as through contact with aborted fetuses and placental membranes and fluids. Bovine brucellosis is transmitted among cattle (*Bos taurus*), bison, and elk, primarily through contact with infected aborted fetuses and placentas and to calves from infected milk.[58] GonaCon has been and is currently being tested to prevent parturition in bison. Studies in bison showed that a single dose of the vaccine resulted in infertility in all vaccinates for multiple years.[50] If ongoing studies demonstrate GonaCon's utility in decreasing shedding of *Brucella abortus*, the vaccine could provide a potential nonlethal management tool to prevent transmission of the disease in an infected population.

GonaCon has been tested as a tool for the treatment and prevention of adrenal cortical disease (ACD) in domestic ferrets.[40] ACD is a common problem in neutered middle-aged and older ferrets, affecting the majority of animals by 7 yr of age, often causing alopecia, adrenal hyperplasia, and tumors. ACD is caused by the excessive continuous production of LH due to the lack of negative feedback from gonadal hormones in neutered ferrets, resulting in overproduction of sex steroids by the adrenal glands. Injection with GonaCon reduces the production of LH, and thus the adrenal sex hormones, thereby reducing ACD and its clinical signs. In an ongoing study, 88 neutered young male and female ferrets were vaccinated with GonaCon.[40] Only 25% of treated ferrets developed clinical signs of ACD by 8 yr of age compared with 84% of control ferrets, indicating that GonaCon may offer a therapeutic tool for the prevention of ACD in ferrets.

SAFETY AND TOXICITY OF GONACON

The NWRC has conducted several studies that have shown that GonaCon can reduce fertility in deer for multiple years without any obvious side effects other than injection site reactions visible upon necropsy. GonaCon is currently registered with the Environmental Protection Agency (EPA) for use in white-tailed deer and in wild horses and burros (GonaCon-Equine). A safety study was designed to evaluate the potential for negative health effects if deer accidentally received multiple injections of GonaCon.[21] In the study, seven does were given a single injection of GonaCon (EPA-registered formulation) and another six does were given three injections at 2-wk intervals. Both groups were compared with a saline control group ($n = 6$). Blood samples were drawn periodically, and the deer were observed for general health. At 20 wk, the deer were euthanized, necropsies were conducted, and the blood was tested using hematology and chemistry health profiles. Aside from some granulomata formation seen at necropsy in the muscle at the injection site, typically evidenced by a tan-yellow appearance and a bulge at the cut surface, there were no toxic or health effects associated either with GonaCon given in a single injection or in multiple injections.

Another potential concern addressed by the NWRC was the health of animals when injected with GonaCon while pregnant. The mechanisms required for the maintenance of pregnancy differ between species; however, in all mammalian species progesterone, from the corpus luteum and or placenta, is required at some stage. In many years of using GonaCon in white-tailed deer, no indications of abortions after treatment were noted; however, to test this, GonaCon was injected into six deer that tested positive for pregnancy by ultrasound in the first week of February. All deer gave birth to healthy fawns but were then infertile for 2 to 3 yr.[43] Other animals have also been injected with GonaCon while pregnant, including elk, bison, and wild horses;[56,50,28] all delivered normal offspring the next year and then were infertile in the following years. Thus, in species tested to date, GonaCon is safe for use in pregnant animals.

Research was also conducted to determine the effect of using GonaCon on white-tailed deer fawns, because it is sometimes difficult to tell a fawn from a doe in the late summer through winter, when darting with an immunocontraceptive vaccine could occur.[43] To determine the effects on fawns, six male and six female fawns were given two injections of 450 μg of GonaCon 1 mo apart at 3 and 4 mo of age, in September and October. All fawns developed initial elevated titers to GnRH; however, the GnRH antibody titers in 11/12 fawns dropped by the following fall, and those fawns came into breeding condition. Only one female retained a high antibody titer and remained infertile throughout the 3-yr study.[43]

Another of the concerns with using an immunocontraceptive vaccine is the occurrence of injection site reactions. Successful stimulation of the immune system requires some level of injection site reaction because the injection site represents the area where the animals' immune system sees and attacks the invading foreign

material, creating an immune response. As discussed, vaccines that are able to remain at the injection site, without being destroyed, can provide a long-lasting immune response. Injection site reactions vary among vaccine formulations, species, and animals. For example, vaccines containing mineral oil-based adjuvants produce more frequent injection site reactions because of the vaccine emulsion remaining at the injection site. If the vaccine is not cleared from the injection site area, the body walls this area off, forming a granuloma. In some cases, more severe reactions occur that result in open sores or draining abscesses.

There are large differences among species in susceptibility to injection site reactions. Some species, including cats,[32] wallabies,[60] and grey kangaroos (*Macropus giganteus*),[60] appear to be less prone to injection site reactions than others after treatment with GonaCon. After a single GonaCon injection, Levy reported no injection site reactions in female cats during the first 2 yr of a study; at 2 yr, the appearance of a nonpainful but persistent late-onset injection site mass was reported in some animals.[32] Lyn Hinds (pers. comm.) also noticed no initial injection site reactions in grey kangaroos or wallabies but observed in some wallabies late-onset nonpainful injection masses (up to 2 cm wide) that would come and go over a period of months in the GonaCon injection site.

In contrast, dogs ($n = 3$) treated with GonaCon (registered formulation; Table 1) experienced severe injection site reactions.[19] This may be due to general differences between species, with dogs being more susceptible to these types of reactions. It is suspect that the amount of *M. avium* in the vaccine is, at least in part, responsible for the injection site reactions in dogs. In a subsequent trial, dogs ($n = 12$) were treated with GonaCon that contained half the amount of *M. avium* than was previously tested. Dogs still experienced injection site reactions, but they were less severe (S. Bender, pers. comm.). A third trial was conducted in Mexico, where dogs were treated using only one-fourth of the *M. avium*.[63] No severe injection site reactions occurred, and there were no signs of pain or limping in the dogs because of vaccination. At necropsy, there was evidence of gross and microscopic lesions in muscle tissue, but reactions were much reduced compared with previous trials. Additional trials are ongoing with GonaCon-Canine, a formulation with the reduced *M. avium* concentration.

In the authors' studies, injection site reactions have occurred more frequently in field trials than in pen trials with white-tailed deer.[13,14] During the 19 yr of vaccinating deer housed in Penn State University pens, there were no visible injection site reactions in any of the deer, and necropsies following the studies did not show any remarkable injection site reaction. These Penn State deer were fed a high-quality diet and were medicated three times a year to ensure they were parasite free. Because of the complete lack of injection site reactions, it was a surprising that some of the deer in the field studies[13,14] developed injection site reactions that were visible upon necropsy. The deer were in poor health because of overpopulation of the deer herd, and at necropsy we found the deer were infested with many parasites. It is possible that poor general health and/or high parasite load may be correlated with an increase in injection site reactions, but further research is necessary to explain the mechanism behind the differences seen.[15]

Similarly, contraceptive efficacy of GonaCon was consistently higher in captive than in free-ranging white-tailed deer and feral horses.[13,14,18] It was hypothesized that parasites in the free-ranging study animals may have reduced their hosts' immune responses to vaccination with GonaCon. Captive animals in these trials received optimal nutrition and regular veterinary care, including routine deworming, whereas free-ranging wild deer and horses received no veterinary intervention. Similar variation in vaccine efficacy has been observed in humans, where several vaccines used in infants failed to confer the expected protection in heavily helminth-parasitized populations but were highly efficacious in populations in which parasitism was uncommon.[15] Abundant experimental evidence from studies in humans and animal models indicates that parasitic helminths can suppress or divert host immune responses and reduce vaccine efficacy. Therefore, inferences on effectiveness of a contraceptive vaccine based on pen studies may be overly optimistic.

DEVELOPING A PHARMACEUTICAL-GRADE VACCINE

To move from a benchtop experimental vaccine to one that could be registered by the EPA and potentially licensed to a manufacturer, the NWRC needed to make changes in some of the GonaCon vaccine inert ingredients and the manufacturing process. Changes included using higher-quality mineral oil and surfactant, moving the

manufacturing process from a chemistry benchtop to a clean room, and developing an aseptic manufacturing technique to deliver a sterile vaccine product.

For GonaCon to be mass-produced for sale, the need for a more efficient and economical production system is needed. Several steps have been made to produce larger GonaCon batches, reduce the cost of reagents, and reduce the time involved in the manufacturing process. Production of GonaCon requires the proper conjugation of GnRH peptides to a mollusk carrier protein and the preparation of a stable water-in-oil emulsion of the GnRH conjugate with the AdjuVac adjuvant. The authors' were able to shorten the time required to make the GnRH conjugate and the amount of reagents used. This allowed the production of a quality emulsion by using a commercial emulsifier machine (Microfluidics, Newtown, Massachusetts, 02464, USA). This helps to reduce variability between batches and allows for the vaccine to be preloaded in syringes, thus eliminating the need to mix vaccines in the field. Quality control steps have been added to the manufacturing process for GonaCon, including a determination of the amount of the active ingredient, the GnRH peptide, in the GonaCon vaccine.[16] Determination of the stability of the active ingredient is required by the EPA for the GonaCon registration, so the chemistry section of the NWRC developed an analytical method to determine the amount of the active ingredient in GonaCon; this method was used to perform a vaccine stability study. In addition to the chemistry analytical method, a biologic assay in rabbits was used to determine efficacy of the GonaCon vaccine. These studies have allowed for a 6-mo shelf life on the outgoing GonaCon vaccine when it is stored and shipped at 4°C.

SUMMARY AND FUTURE DIRECTIONS FOR NWRC REPRODUCTIVE CONTROL RESEARCH

During the 20 yr that the NWRC has been conducting research on immunocontraceptives, the NWRC has had successes and failures and has learned some important general lessons. Both PZP and GnRH antigens can be effective antigens for immunocontraceptive vaccines in many species. Each of these products has advantages and disadvantages, and its use needs to be based on evaluation of the wildlife problem to be solved. The effectiveness of the vaccines is dependent on their formulation and on the proper presentation of the target antigen. The vaccines require a powerful adjuvant and a water-in-oil emulsion to be most effective. The presence of a ubiquitous *Mycobacterium* (killed) in the adjuvant enhances vaccine efficacy.

When used in combination with AdjuVac, SpayVac (a PZP vaccine), and GonaCon (a GnRH vaccine), formulations can provide single injection, multiple year effectiveness. A boost vaccination can extend the effectiveness of the vaccines, sometimes making the animal permanently infertile, but the effectiveness of vaccines varies between species and among individuals. A certain proportion of animals will be nonresponders and will not generate a high antibody titer. Vaccines are most effective if injected at least a month or more before the onset of reproductive activity to allow time for antibody development. Field efficacy of a vaccine may be lessened by high parasite load and poor body condition. Localized, nonpainful injection site reactions caused by the long-term presence of the vaccine can occur but may be part of the process of developing a long-lasting antibody response after a single injection. Animals treated with either PZP or GonaCon vaccines can be consumed because the protein antigens are broken down into amino acids in the gastrointestinal tract.

In addition to these general lessons, the NWRC has gained considerable insight into PZP vaccines. For example, the native PZP was shown to be much more antigenic than peptides or recombinant proteins. Also, PZP vaccines are not effective in all mammalian species.[17] PZP vaccines may cause undesirable behavioral effects such as multiestrus.

During its development and testing, the NWRC has observed that GonaCon is effective in most mammalian species, with contraceptive effects lasting longer in females than in males. GonaCon can be safely given to pregnant animals without interfering with the pregnancy. GonaCon can eliminate almost all reproductive behaviors, including estrus cycles and theoretically some undesirable behaviors in companion animals. It may also provide a potential management tool for venereally transmitted diseases or diseases transmitted at parturition. GonaCon can effectively prevent ACD in domestic ferrets by reducing LH levels. However, approval for use of GonaCon in companion animals or for disease prevention will be through regulatory agencies other than the EPA and will not be pursued by the Animal Plant Health Infection Service.

Future research at the NWRC will continue to look for new applications for the use of GonaCon and will continue research into understanding the immunology behind the variations in effectiveness from species to species. It is hoped that information on the timeframe in which to give a boost to improve the length of the contraceptive response can be developed. The NWRC also hopes to pursue studies on its effective use as a management tool and to develop private sources of manufacturing for GonaCon in efforts to commercialize the product and make it more widely available.

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