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Effects of the Immunocontraceptive Gonacon on Pregnancy in *Brucella*-Seropositive American bison (*Bison bison*)

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ABSTRACT: The purpose of this study was to determine if the number of pregnancies in naturally infected *Brucella abortus*–positive bison (*Bison bison*) cows would be reduced over a period of 5 yr after one treatment with 3000 μg gonadotropin-releasing hormone immunocontraceptive (GonaCon) compared to a similar group of naturally infected *B. abortus*–positive bison cows not treated with GonaCon. In each of the 5 yr, GonaCon-treated cows produced fewer offspring in relation to number of cows than the nontreated cows. Fisher's Exact test comparing offspring produced during the first reproductive season showed a significant difference between the two groups (P=0.0028). Differences in number of calves produced in GonaCon-treated and control groups were also noted in remaining years, but statistics were not applied because of data constraints. These data indicate that one treatment with GonaCon in brucellosis-seropositive female bison reduced pregnancies over five reproductive years. Thus, immunocontraception could potentially be used to manage brucellosis in affected herds.

Key words: Bison, *Brucella abortus*, immunocontraception, GnRH, GonaCon, pregnancy.

INTRODUCTION

Brucellosis is a reproductive disease of bovids and other ruminant species caused by the bacterium *Brucella abortus*. The primary mode of transmission of this organism is through mucosal contact by susceptible animals with tissues and fluids associated with abortions and infectious calving events (Rhyan et al. 2001). Brucellosis was first reported in the early 1900s in the wild bison (*Bison bison*) herds of the Greater Yellowstone Area (GYA), and much effort and resources have been expended to maintain temporal and spatial separation between wild bison herds and domestic livestock in the GYA as well as to apply lethal removal of brucellosis test-positive bison (Mohler 1917; National Academies of Sciences, Engineering, and Medicine 2020). These tools, while feasible, are not necessarily a long-term disease management solution. Adaptive management through the Interagency Bison Management Plan (IBMP) has been used to advance efforts based on new and novel techniques (United States Department of the Interior, National Park Service, and United States Department of Agriculture 2000). Immunocontraception is one such technique that has been proposed as a management tool for brucellosis in wild bison (Rhyan and Drew 2002).

A single dose of the immunocontraceptive GonaCon (USDA, APHIS, Wildlife Services, National Wildlife Research Center, Fort Collins, Colorado, USA), a gonadotropin-releasing hormone (GnRH) -based immunocontraceptive licensed for use in wild deer and feral horses, has been shown in two previous studies in bison

to be effective in preventing pregnancy for more than 5 yr and 6 yr in 83% and 100% of the treated animals, respectively (Rhyan et al. 2013). However, the purpose of our current study was to determine if one treatment with GonaCon would decrease pregnancies in a group of naturally infected *B. abortus*–positive bison cows as compared to a similar group of bison cows not treated with GonaCon over 5 yr.

MATERIALS AND METHODS

Animals

We selected and acquired 39 nonpregnant, 1–2-yr-old female bison of both *Brucella*-seropositive (29) and *Brucella*-seronegative (10) status during IBMP removal operations near the northern boundary of Yellowstone National Park in 2011. Animals were captured at the National Park Service's Stephens Creek, Montana, USA (45°2′56.76828″N, −110°45′4.76568″W) bison facility, as they migrated out of Yellowstone National Park (YNP) in late winter and early spring of 2011. We also collected ten 2-yr-old and 3-yr-old *Brucella*-seronegative bulls from IBMP operations at Stephen's Creek (2012–14) and additionally (2012) purchased four *Brucella*-seronegative bulls from a commercial source. Bison were manually restrained in a chute, then blood was collected via jugular venipuncture using a 16-gauge needle and 30 mL syringe for chute-side brucellosis screening using a fluorescence polarization assay (FPA) and the standard card test (United States Department of Agriculture, Animal and Plant Health Inspection Service, 2003). Bison were individually identified with visual ear tags (Y-Tex, Cody, Wyoming, USA) and a radio frequency identification button tag (AllFlex USA, Dallas Fort Worth Airport, Texas, USA). Animals were transported to the USDA, Animal and Plant Health Inspection Service (APHIS) bison facilities in Corwin Springs, Montana, USA (45°6′49″, −110°47′248″). We randomly assigned animals to a control group not treated with GonaCon (*Control*; n=14) or a treatment group that received one dose of 3000 µg GonaCon as described below (*Treatment*; n=15). Each group was housed separately in an approximately 10 ha pasture. In addition, each pasture also contained five *Brucella*-seronegative cows that served as a comparison of fertility to brucellosis-seropositive animals during the first year, as well as disease transmission sentinels for an associated study (Nol et al. 2024). *Brucella*-seronegative cows did not receive GonaCon.

Bison bulls were housed separately from the cows until breeding season. Bulls (two per pen) were comingled with cows from August to October. Commercially sourced bulls were removed from the study after the first breeding season.

GonaCon production and treatment

GonaCon was prepared using GnRH-blue protein hemocyanin conjugate and adjuvant formed into an emulsion, for depot effect, as described previously (Miller et al. 2008), with some differences in emulsification. Specifically, GonaCon was emulsified by passing it once through a microfluidizer processor run at 6,000 psi with an H30Z interaction chamber (Microfluidics M110L, Microfluidics, Westwood, Massachusetts, USA). At initiation of the study (2012), *Treatment* animals were manually handled in a chute and injected intramuscularly (IM) 2.5 cm from the hip, bilaterally with GonaCon (total of 3000 µg GnRH-blue protein conjugate in 2 mL adjuvant; 1500 µg in 1 mL was administered in each hip) approximately 90 d before first exposure to bulls. GonaCon was administered only one time to each individual treatment animal. *Control* bison were not treated with GonaCon, nor were *Brucella*-seronegative animals in either group.

Animal handling and sample collection

Each January, for five reproductive seasons, we manually restrained bison cows in a chute, during which time we collected blood and determined pregnancy status by rectal palpation. Blood samples were collected from the jugular vein as described above, transferred to serum separator tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA), and submitted to the Montana Veterinary Diagnostic Laboratory, Bozeman, Montana, USA (MVDL), for brucellosis testing as described (Nol et al. 2024). Animals determined to be pregnant immediately received a vaginal transmitter (Advanced Telemetry Systems Inc., Isanti, Minnesota, USA) and were monitored daily thereafter for birth events. Blood was also collected from cows either directly post-partum or midyear (nonpregnant animals). Blood was collected from bulls annually just prior to breeding for brucellosis testing. We evaluated all bulls for breeding soundness over the course of the study.

Electroejaculation (Lane Manufacturing Inc., Denver, Colorado, USA) of bulls was performed using warmed collection cups or tubes to avoid cold shock to the sperm. We evaluated and scored bull semen samples for gross and individual motility using Society for Theriogenology (SFT) -published criteria (Koziol and Armstrong 2018).

For post-partum handling of cows and breeding soundness examinations for bulls, animals were chemically immobilized using etorphine (0.01 mg/kg) or thiafentanil (0.015–0.02 mg/kg) and xylazine hydrochloride (0.05–0.07 mg/kg; Wildlife Pharmaceuticals, Windsor, Colorado, USA) delivered IM via remote injection (Pneudart, Williamsport, Pennsylvania, USA). Immobilizing drugs were antagonized with naltrexone (Wildlife Pharmaceuticals; 50 mg IM per mg thiafentanil given or 25 mg IM per mg etorphine given) and 300 mg IM tolazoline (Akorn Animal Health, Lake Forest, Illinois, USA; 300 mg, half IM, half subcutaneously).

Brucellosis testing

Serologic testing by standard card, buffered acidified plate antigen, complement fixation, and FPA as described in the *Uniform methods & rules* for brucellosis eradication (United States Department of Agriculture, Animal and Plant Health Inspection Service, 2003) were performed at the MVDL, both on animal acquisition (for confirmatory testing of chute-side results) and for the remainder of the study.

Anti-GnRH antibody testing

We measured anti-GnRH antibodies using ELISA as follows. Microtiter plates (Greiner Bio-One North American Inc., Monroe, North Carolina, USA) were coated using 50 µL of a solution containing 4 ng GnRH-BSA (bovine serum albumin) conjugate in carbonate-bicarbonate buffer. Plates were incubated at 4 C overnight, then washed three times with 300 µL of 0.01 M phosphate-buffered saline (PBS) plus 0.05% Tween 20 (pH 7.4; giving PBST) per well at room temperature. To block nonspecific binding, 200 µL of a solution consisting of 20% (v/v) SeaBlock (Thermo Fisher Scientific; Waltham, Massachusetts, USA) and 5% (v/v) Tween 20 in 0.01 M PBS was added to each well, and plates were incubated for 1 h at 25 C, followed by another three washes with PBST. Serum samples were run in duplicate at a dilution of 1:2,000 in 50 µL of 0.01 M PBS and incubated for 1 h at room temperature. Plates were then washed three times with PBST. Rabbit anti-bovine IgG (Sigma-Aldrich, Inc., St. Louis, Missouri, USA), diluted 1:5,000 in 0.01 M PBS (50 µL), was added to each well and incubated for 1 h at 25 C, followed by two washes with PBST. Secondary antibody, goat anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP) (Sigma-Aldrich), diluted 1:3,000 in 0.01 M PBS (50 uL), was added to each well and incubated for 1 h at 25 C, followed by two washes with PBST. Enzyme substrate (3,3′,5,5′-tetramethylbenzidine dihydrochloride) in 0.05 M phosphate citrate buffer with 0.14% urea hydrogen peroxide was then added to each well. After 3 min, 50 µL of 2 M sulfuric acid was added to terminate the reaction. Absorbance of each well was measured at 450 nm on a plate reader (VarioSkan Flash, Thermo Fisher Scientific). Plate background was corrected by subtracting the mean absorbance of all PBS wells from all other well values. Antibody responses were reported as optical densities (ODs). A positive control sample was included on each plate. Linearity was determined from eight concentrations of positive serum ranging from 1:1,000 to 1:128,000. The R^2 value was 0.98. The dilution used (1:2,000) to measure antibody response was at the upper end of the working range. Positive and negative control samples were included on each plate. The interassay coefficient of variation for the positive controls was 12%.

Data analysis

We limited the statistical analysis of pregnancy data to year 1. We limited statistical analysis of OD data to baseline (pre-treatment), 8 mo, and 44 mo post-treatment. Both sets of data were truncated due to potential bias issues resulting from missing values, animal removals, and, specific to the ELISA, unexplained output irregularities in other time points for all animals regardless of treatment. For the remaining data we compared differences between the treatment groups in number of calves produced and OD values of anti-GnRH antibodies using the methods described below.

The number of pregnancies or calves produced, alive or dead, was the variable of interest in evaluating GonaCon in its ability to prevent pregnancy. We used a Fisher's exact test, given the small sample size, to compare the number of calves produced between *Treatment* and *Control*, in year 1 only as stated above. Comparisons of anti-GnRH OD between *Treatment* and *Control* were conducted using a repeated measure ANOVA for three measurement periods:

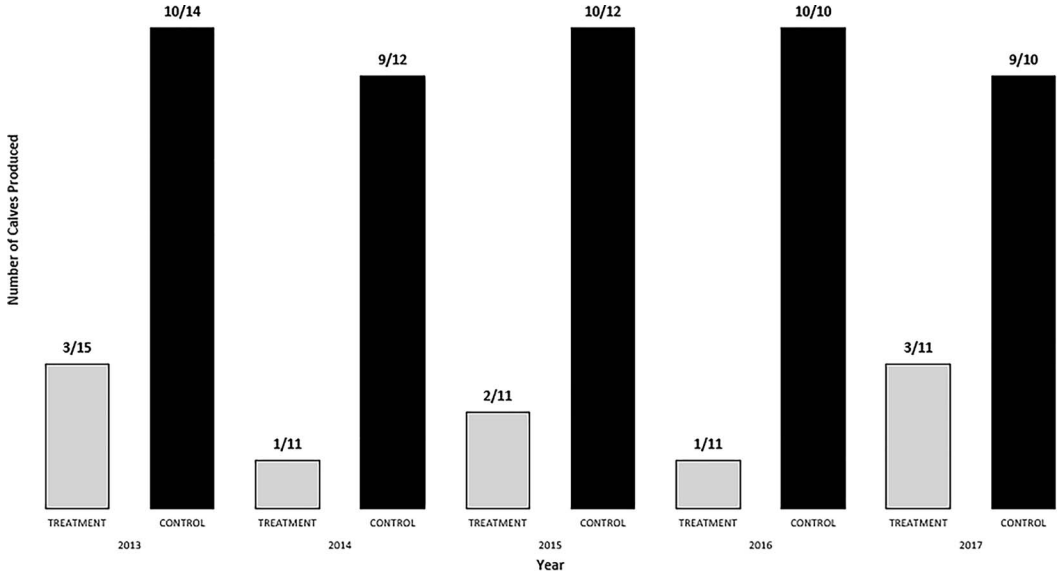

FIGURE 1. Comparison of number of calves produced per number of bison (*Bison bison*) cows (calves/cows) in bison cows treated with GonaCon versus nontreated bison cows between 2013 and 2017. GonaCon-treated bison cows received a one-time initial dose of 3000 μg gonadotropin-releasing hormone immunocontraceptive (GonaCon) on initiation of the study and were tracked over 5 yr for evidence of pregnancy. All bison cows (both Gonacon-treated and nontreated) were brucellosis-positive. A pregnancy was recorded as such regardless of calf survival outcome. Cow numbers varied over time because of mortalities and other experimental attritions.

baseline, 8 mo post-treatment, and 44 mo post-treatment. Pairwise tests with Bonferroni correction were run to detect any differences between the within-treatment time periods. Prior to conducting the repeated measures, data were checked for normality using a Shapiro-Wilk's test and QQ plots. Values were considered significant at $P=0.05$. All statistical tests were performed in R (R Core Team 2022).

RESULTS

In GonaCon-treated bison, no adverse effects were observed. Mortalities unrelated to treatment did occur, and three animals were removed from *Treatment* (related to another study) after the first year, resulting in fewer animals ultimately completing the study. Four *Control* cows died, and one *Treatment* cow died over the 5-yr period, resulting in 10 cows comprising the *Control* group and 11 cows comprising the *Treatment* group by the final year (Fig. 1).

Over all five years, *Controls* produced 50 calves, while the GonaCon-treated animals produced only 10 calves. In each of the 5 yr that animals were monitored, GonaCon-treated animals produced fewer offspring in proportion to cow numbers than the nontreated animals (Fig. 1). Fisher's Exact test comparing offspring produced during the first reproductive season showed a significant difference between the two groups ($P=0.0028$). Data after the first year were not analyzed statistically, although differences diminished over time.

There were no statistical differences between *Treatments* and *Controls* in baseline and 44-mo anti-GnRH OD values, but at 8 mo post-treatment the two groups differed ($P\leq0.0001$). Within *Treatment* group comparisons at baseline and 44 mo values did not statistically differ, but OD values at 8 mo post-treatment differed from baseline ($P\leq0.0001$). Within the *Control* group, 8-mo and 44-mo values were also significantly different from baseline ($P=0.03$; Fig. 2).

Bulls were never seropositive on brucellosis testing throughout the study. All bulls were found to have adequate numbers of motile sperm with normal morphologies in their ejaculates (data not shown; J. Barfield, pers. comm.).

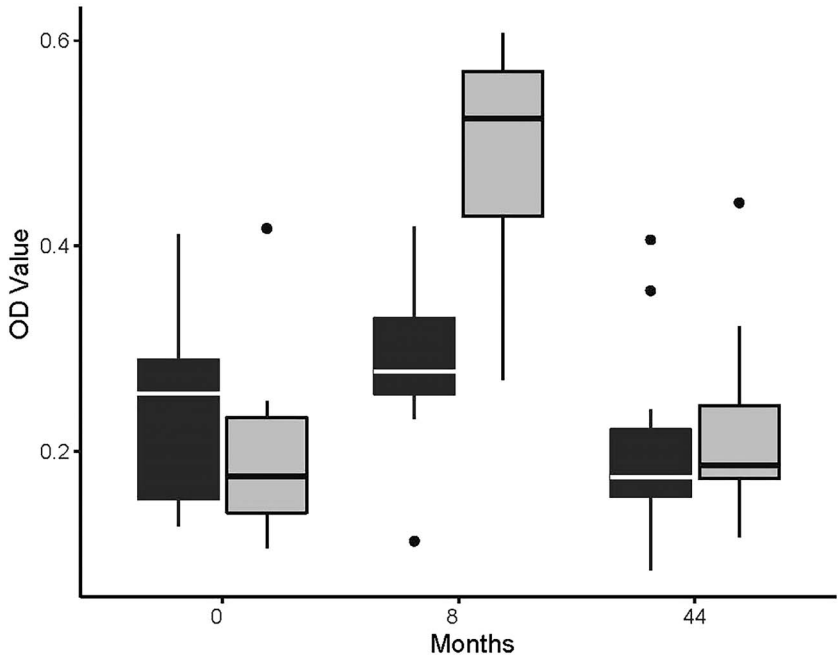

FIGURE 2. Box plots illustrating results from repeated measures analysis of variance of anti-GnRH antibody optical densities (ODs), detected by ELISA, in serum collected from GonaCon-treated bison (*Bison bison*) cows and nontreated bison cows at baseline (0 mo), 8 mo post-treatment, and 44 mo post-treatment. GonaCon-treated cows received a one-time dose of 3000 µg GonaCon on initiation of the study. Dark gray boxes represent median OD values (horizontal line) plus the upper and lower quartile values in GonaCon-treated bison cows. Light gray boxes represent median OD values (horizontal line) plus the upper and lower quartile values in non-treated cows. Significant differences in OD occurred at 8 mo between Gonacon-treated and non-treated cows (repeated measures ANOVA; $P \leq 0.0001$), and at 8 mo and 44 mo in non-treated cows as compared to baseline ($P=0.03$). Outliers in data are depicted by small dots for each time frame and group.

DISCUSSION

Our data show that a group of brucellosis-positive bison cows given one administration of 3000 µg intramuscular GonaCon prior to breeding will produce fewer calves than untreated brucellosis-positive bison over a period of 5 yr. GonaCon is thus effective at reducing pregnancies in these animals, although the effect does diminish over time. We have observed this also in a parallel study with *Brucella*-seropositive bison where 14/19 GonaCon-treated cows did not become pregnant over a 3-yr period (Nol et al. 2024). Data from previous studies showed GonaCon to be more effective in preventing pregnancy in the first reproductive season after treatment than what we observed in this study; however, in subsequent years post-treatment, GonaCon in this study performed similarly to previous studies (Miller et al. 2004; Schoenecker et al. 2019). Rhyan et al. (2013) was the exception, in which GonaCon appeared to induce sterility in all animals in the 6 yr that they were observed after GonaCon treatment. Eight cows in our *Treatment* group remained infertile throughout the five reproductive seasons. As some treated bison in this study did return to normal reproductive cycles, this may alleviate some concerns of permanent sterility in these animals.

The number of pregnancies in nontreated cows, including the originally seronegative animals, was consistent with the observed pregnancies for animals of this age group in Yellowstone National Park (C. Geremia, pers. comm.; Fuller et al. 2006). The bulls were also tested for possible

effects on pregnancy. While not every bull had a complete breeding soundness exam every year, all of the bulls were evaluated over the course of the study and deemed reproductively sound (fertile). Since bulls were rotated among the control and treatment groups each year, it seems unlikely that a bull effect caused any differences in numbers of pregnancies. In addition, none of the bulls became *Brucella*-seropositive during the study, so no brucellosis effects on fertility of bulls would have interfered with breeding.

The outcome of the ELISA data analysis was unexpected because *Treatments* were still producing fewer calves than *Controls* at that time. This might reflect the anti-GnRH ELISA limitations, or perhaps cellular immunity mechanisms are activated that we did not anticipate or look for. Given the overall lower proportion of pregnancies in the *Treatment* group, it seems clear that GonaCon was efficacious in this case.

The outcome of this study is useful because reduction of pregnancy might effectively reduce spread of *B. abortus* in affected herds. Other factors also can contribute to the spread of *B. abortus*, such as population density, but reducing reproductive fluids and tissues on the landscape directly addresses the primary mode of transmission (Rhyan et al. 2009).

Use of GonaCon to reduce pregnancy among *B. abortus*–infected bison should be further investigated as a nonlethal tool to control the spread of brucellosis. This may enable reduced spread of the disease while preserving valuable genetics in the population, as the treated animals may eventually come back into estrus and successfully produce a live calf. As the Yellowstone bison population continues to expand, tolerance for the bison on a larger landscape could be greater if the number of infected animals is decreased.

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