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Concomitant administration of GonaConTM and rabies vaccine in female dogs (*Canis familiaris*) in Mexico[☆]



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ABSTRACT

Mexico serves as a global model for advances in rabies prevention and control in dogs. The Mexican Ministry of Health (MMH) annual application of approximately 16 million doses of parenteral rabies vaccine has resulted in significant reductions in canine rabies during the past 20 years. One collateral parameter of rabies programs is dog population management. Enhanced public awareness is critical to reinforce responsible pet ownership. Surgical spaying and neutering remain important to prevent reproduction, but are impractical for achieving dog population management goals. GonaConTM, an anti-gonadotropin releasing hormone (GnRH) vaccine, was initially tested in captive female dogs on the Navajo Nation, 2008. The MMH led this international collaborative study on an improved formulation of GonaConTM in captive dogs with local representatives in Hidalgo, Mexico in 2011. This study contained 20 bitches assigned to Group A (6 control), Group B (7 GonaConTM), and Group C (7 GonaConTM and rabies vaccine). Vaccines were delivered IM. Animals were placed under observation and evaluated during the 61-day trial. Clinically, all dogs behaved normally. No limping or prostration was observed, in spite of minor muscle atrophy post-mortem in the left hind leg of dogs that received GonaConTM. Two dogs that began the study pregnant give birth to healthy pups. Dogs that received a GonaConTM injection had macro and microscopic lesions consistent with prior findings, but the adverse injection effects were less frequent and lower in intensity. Both vaccines were immunogenic based on significant increases in rabies virus neutralizing antibodies and anti-GnRH antibodies in treatment Groups B and C. Simultaneous administration of GonaConTM and rabies vaccine in Group C did not affect immunogenicity. Progesterone was suppressed significantly in comparison to controls. Future studies that monitor fertility through multiple breeding cycles represent a research need to determine the value of integrating this vaccine into dog rabies management.

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1. Introduction

Mexico is a global model for advances in rabies prevention and control in dogs (*Canis familiaris*). During the past 20 years the Mexican Ministry of Health (MMH) has applied approximately 16 million doses of parenteral canine rabies vaccine annually, resulting in a substantial reduction in canine rabies [1,2]. This accomplishment not only illustrates the effectiveness of these campaigns, but also brings the elimination of canine rabies into focus as a potentially achievable goal in developing countries. However, effective dog population management is central to achieving that goal. Clearly, enhanced public awareness is critical to reinforce the key role of responsible pet ownership to reduce the risks associated with dog overpopulation to human and animal health. Spaying and neutering remain important components to prevent unwanted reproduction, but they are expensive, invasive, time consuming and impractical for achieving broader dog population management goals integral to canine rabies elimination [1,3]. Fertility control holds promise as an efficient and less invasive method in rabies management in Mexico and perhaps globally.

México maintains a close relationship with the U. S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) through the North American Rabies Management Plan, signed by representatives of Canada, Mexico, the Navajo Nation and the U.S. in 2008. This plan serves as a basis for collaborations to develop, study and implement methods and strategies to enhance rabies management. The immunocontraceptive vaccine GonaCon™ represented an ideal candidate for evaluation in captive dogs in Mexico [4,5]. Results from this study should provide a foundation for continued evaluation of GonaCon™ as a prospective method for dog population management.

The MMH and USDA, APHIS, Wildlife Services (WS), with technical support of many partners, collaborated in a study of a new formulation of GonaCon™ in captive dogs in Mexico. The study was conducted in Hidalgo State in collaboration with State Health Services, and invited institutions and groups. The primary objectives were to: (1) determine the immunogenicity of simultaneous IM administration of GonaCon™ and rabies vaccine and (2) assess potential adverse health effects in dogs, including injection site reactions from this new vaccine formulation.

2. Materials and methods

2.1. GonaCon™ and canine rabies vaccine

GonaCon™ is an immunocontraceptive vaccine (USDA, APHIS, Wildlife Services (WS), National Wildlife Research Center (NWRC), Fort Collins, CO, USA) consisting of mammalian gonadotropin releasing hormone (GnRH) conjugated to a large mollusk hemocyanin protein and emulsified with the adjuvant AdjuVac™. Unlike prior GonaCon™ formulations, the one used in this study was produced in a clean room to reduce the chance of particulate contamination and Gentamicin and Fungizone (Life Technologies, Carlsbad, CA) were added to inhibit bacterial and fungal activity. Each 0.5 ml dose contained 500 µg of the GnRH conjugate and 21 µg of inactivated *Mycobacterium avium* in the adjuvant. GonaCon™ was stored and shipped under refrigerated conditions in pre-loaded, 3 ml Air-Tite luer-lock syringes (Air-Tite Products, Virginia Beach, VA, USA) [5].

A commercial inactivated rabies virus vaccine was administered (Rabiffa, Merial Inc., El Marques, Qro., Mexico) that is commonly used by the State Health Services during the National Weeks of Dog Rabies Vaccination in accordance with Mexican Regulations. By special request from the MMH, the rabies vaccine was supplied



Fig. 1. Stray dogs housed and cared for at PROVEZA's Shelter and Refuge (Cerrada 16 de Septiembre s/n Colonia Centro, Villa de Tezontepec, C.P. 43882, Hidalgo, Mexico) included in Group B of the GonaCon™ study in Mexico 2011.

Photo taken by Dr. Luis Lecuona.

in 20 ml vials containing 20 dosages (1 ml/dose), stored at 4 °C until ready for use [6].

2.2. Vaccine administration

Vaccines were administered once IM throughout this study in the upper right hind leg (rabies vaccine) or upper left hind leg (GonaCon™). A 3 ml disposable syringe and 21 G × 38 mm needle were used to administer vaccines.

2.3. Experimental animals

Twenty female dogs of mixed breeds were collected from September to November 2010 for this trial. Six owned bitches came from the Villa de Tezontepec County, in Hidalgo State. The 6 owners signed an authorization for inclusion of their dogs in the study. Fourteen other bitches were collected at dog round-ups conducted by the Canine Attention Centers from the Sanitary Regions of Pachuca and Tulancingo in Hidalgo State. Typically, unclaimed dogs at the Canine Attention Centers are euthanized in a 48–72 h post round-up pursuant to the National Animal Control Regulation (NOM-042-SSA2-2006. Number 4.2.14, Section 4.2.) [7]. Fourteen bitches over 1 year old were selected from this source for this study. Dogs were selected based size (medium or large), apparent healthy clinical status and general body condition. Dogs were held for a 60-day observation and adaptation period.

Six bitches in Group A were confined at the home of their owner. The other 14 bitches were divided into vaccination Group B (GonaCon™) or Group C (GonaCon™ and rabies). These groups included 7 animals maintained in communal kennels in the PROVEZA's Shelter and Refuge (Cerrada 16 de Septiembre s/n Colonia Centro, Villa de Tezontepec, C.P. 43882, Hidalgo, Mexico; 19° 52' 45.33 N, 98° 49' 13.36' W) (Fig. 1). This facility offered complete dog holding support during this study. Each bitch was marked with a collar and unique numbered tag for identification. Animal care personnel maintained the kennels twice/day. Dogs were fed a commercial dog food (Adult Natural Balance Pedigree, Mars Inc., Mexico) and water ad libitum. All female dogs were evaluated by veterinarians, State Health Services, Hidalgo during the 61-day study.

The 6 owned bitches chosen for Group A were vaccinated with the rabies vaccine. The 14 female dogs maintained in the PROVEZA's Shelter and Refuge were randomly assigned to two treatment groups. Group B included 7 dogs that were vaccinated

with GonaCon™ immunocontraceptive vaccine. Group C included 7 dogs that were simultaneously vaccinated with GonaCon™ and the rabies vaccine. The 61-day observation period for dogs during the trial ended on March 11, 2011.

2.4. Blood samples collection

Blood samples were obtained on days 0, 31 and 61. Blood was drawn from the external jugular vein using a 10 ml disposable syringe with a 20 G × 38 mm needle, or from the radial vein using BD Vacutainer™ 13 mm × 75 mm tubes, with 20 G needles. Samples for complete blood counts (CBC) were collected in EDTA BD Vacutainer™ tubes. Samples for blood chemistry profiles (BCP), progesterone (THR), rabies virus neutralizing antibodies (VNA) and anti-gonadotropin releasing hormone (GnRH) antibodies were collected in SST BD Vacutainer™ tubes for serum separation.

Blood samples were stored on ice in an insulated container until their arrival at the Clinical Laboratory of the Tizayuca's Sanitary Region in Hidalgo State. Samples for CBC and BCP were processed in a 4-h period. Samples for THR, VNA and GnRH were centrifuged. Serum was separated and stored in CryoTubes™ (Nalge Nunc International, Rochester, NY, USA) at -70°C until analyzed.

2.5. Clinical evaluation of the dogs

Veterinary Personnel from the Hidalgo State Health Services performed daily and weekly clinical evaluations of dogs.

2.6. Determination of CBC and BCP

Dogs were evaluated for CBC and BCP as indicators of their health status [8]. For CBC, 6 values were considered: hematocrit value (HCT), hemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), total leukocyte volume (LEU) and platelet count (PLT). These values were determined utilizing a Coulter Counter (Coulter S Plus IV., Coulter Electronics Inc., Hialeah, FL 33010, USA). For BCP, 5 values were considered: glucose (GLU), blood urea nitrogen (BUN), creatinine (CREA), Uric Acid and Cholesterol. All were determined with a multichannel biochemical analyzer (Discrete Analyzer Continues Optical Scanner [DACOS], Coulter Corporate Communications, Hialeah, FL, USA).

2.7. Determination of THR

Progesterone levels were determined at the NWRC. Plasma progesterone levels were assayed by the coat-a-tube RIA method (Diagnostic Products, Los Angeles, CA, USA). The antigen-antibody complex is determined with a gamma spectrophotometer [9].

2.8. Determination of rabies VNA titers

Rabies VNA titers were determined by the National Center of Animal Health Diagnostic Services (CENASA/SENASICA/SAGARPA). The Fluorescent Antibody Virus Neutralizing (FAVN) test, an adaptation of the Rapid Fluorescent Focus Inhibition Test (RFFIT) [10,11], was conducted on tissue culture 96-well microplates using BHK21-13s (ATCC CCL-10) cells and the CVS 11 strain of rabies virus (ATCC VR 959). A virus titer of 10 TCID₅₀/ml. EMEM-10% FCS was used as a diluent for control and test sera, and for the challenge virus. Serial 3-fold dilutions of the positive and negative serum controls and test sera were prepared in microplate wells in 0.1 ml volumes. The well was considered negative if no fluorescent cells were observed. The well was considered positive if one or more fluorescent cells were observed. The serum titers were expressed in I.U./ml by comparison with the titer of the standard serum in each test. The validity of each test was then assessed by recording results obtained from titrations of CVS (TCID₅₀), naive serum (D50) and the positive standard

(D50) on control charts. Each test was considered valid if the values found for all of these controls were not statistically different from the respective means of the values obtained from all previous tests [12,13]. The FAVN end point was adjusted to 13.8 I.U./ml.

2.9. Determination of anti-GnRH titers

Anti-GnRH titers were determined at the NWRC. An enzyme-linked immunosorbent assay (ELISA) was used to measure anti-GnRH antibody titers. A 96-well plate was prepared by adding 100 ng of BSA-GnRH antigen to each well and then blocking with SeaBlock™. Fifty microliters of serially diluted serum was used for each assay. Anti-GnRH antibody was determined by adding rabbit anti-dog IgG to each well, washing, then adding goat anti-rabbit IgG labeled with horse-radish peroxidase (HRP), washing, then developing a color by adding a HRP substrate. The color was proportional to the anti-GnRH antibody titer. BSA-GnRH was added to the ELISA plate, causing only antibodies to GnRH to be detected [14].

2.10. Post-mortem evaluations

Macro and microscopic reactions to GonaCon™ were evaluated for 17 female dogs euthanized to characterize potential variability. The sample included 6 dogs that served as GonaCon™ controls for macro and microscopic effects. In addition, 5 of the 7 female dogs from Group B and 6 of the 7 female dogs from Group C were included in the sample. Procedures recommended [15–17] and established by the Mexican Official Regulation (NOM-033-ZOO-1995. CONASA/SAGARPA) [18] were applied. All dogs were sedated with Xylazine 2% (4.4 mg/kg, 20 mg/ml. Porcin™, PISA Agropecuaria SA de CV. REG. SAGARPA Q7833-009) administered intramuscularly to the upper right hind leg with a disposable 3 ml syringe and 21 G × 38 mm needle. Under sedation, an overdose of pentobarbital (over 40 mg/kg, 0.063 gr/ml, Sedalparma™, Pet's Pharma de México, S.A. de C.V. REG. SAGARPA Q-7972-004) was applied in the radial vein of the right forearm using a 10 ml disposable syringe with a 20 G × 38 mm needle. Necropsies were performed according to routine standards [19].

During necropsy samples taken from skeletal muscle at the GonaCon™ injection site, ovaries and pituitary glands were fixed in formaldehyde (4% PBS). Fixed tissues were embedded in paraffin, prepared in 5 μm sections on slides and stained with H&E [20]. Slides were analyzed with an Olympus BX41 microscope; images were captured with a digital ProEvolution Camera in Image-Pro Express 6.0 (MediaCybernetics).

2.11. Statistical methods

MINITAB Statistical software was used (MINITAB INC 13.1). A 1-way, unstacked ANOVA and Turkey's test were used for the analysis. Statistical significance was set at $P < 0.05$ [21].

3. Results

3.1. Clinical evaluation

Dogs in Group A were clinically healthy. One of 6 dogs showed external vaginal swelling (heat) at D58. No physical abnormalities, prostration, injuries or limping were reported. Two dogs in Group A finished the study pregnant. Average weight on D0 was 21.2 ± 10.5 kg(SD); on D61 it was 21.3 ± 10.7 kg(SD).

During week 1, 4 of 7 dogs (57.1%) in Group B showed signs of pain based on an extension and flexing test of the left leg and hyperthermia in the left leg. Pain began to subside during week 1 and was not noticeable by week 2. One dog had puppies before the beginning of the study. Two dogs had puppies during week 1.

Table 1
Complete blood counts (CBC) and blood chemistry profiles (BCP) in female dogs by Group and Day.

Group/Day	Group A			Group B			Group C		
	D0	D31	D61	D0	D31	D61	D0	D31	D61
CBC									
Erythroblastopenia	2	3	0	1	0	0	0	0	0
Leukocytosis	1	0	0	0	0	0	1	0	0
Leukopenia	1	1	0	7	2	1	0	2	1
Thrombocytopenia	6	1	2	7	1	4	6	0	3
Polycythemia	0	0	2	0	1	3	1	0	2
BCP									
Hypoglycemia	4	1	0	1	1	0	3	0	0
Hyperazotemia	6	6	5	5	6	7	7	7	7
Hypercholesterolemia	1	0	1	0	0	0	0	0	0
Hypocholesterolemia	0	0	0	0	2	0	0	2	0
Hypouremia	0	0	0	0	0	0	0	0	2

Table 2
Mean and standard deviation for progesterone (THR ng/ml) in female dogs by Group and Day.

Group/Day	Group A			Group B			Group C		
	D0	D31	D61	D0	D31	D61	D0	D31	D61
<1.0	2	4	1	5	7	7	3	7	7
=1.0	3	1	0	1	0	0	0	0	0
>1.0	1	1	5	1	0	0	4	0	0
Statistical findings									
Mean	1.71	5.82	31.04	0.90	0.08	0.23	7.52	0.31	0.26
St dev	2.98	14.14	54.69	1.75	0.14	0.31	12.48	0.23	0.32

One dog had a small swelling at the GonaCon™ application site beginning on D16 until the end of the study. All dogs concluded the study with minor muscle atrophy in the left leg that was perceptible by clinical evaluation; however, no prostration or limping was reported. Average weight on D0 was 15.3 ± 5.3 kg(SD); on D61 it was 13.9 ± 3.4 kg(SD).

Not unlike Group B, 2 of 7 female dogs (28.5%) in Group C showed similar signs of pain and hyperthermia in the left leg. Again, pain was not noticeable by week 2. One dog had external vaginal swelling only at D16 and D59, unrelated to heat. One dog had slight swelling at the GonaCon™ application site beginning at D16 that persisted through the study. All dogs concluded the study with minor muscle atrophy in the left leg that was perceptible by clinical evaluation, but no prostration or limping was reported. Average weight on D0 was 15.7 ± 4.6 kg(SD) on D61 it was 15.9 ± 4.1 kg(SD).

3.2. CBC and BCP

All dogs had one or more clinical and metabolic changes in CBP and BCP. This is noteworthy given visible clinical changes were not observed; all dogs had suggestions of chronic disease and metabolic deficiencies (Table 1).

3.3. Progesterone

At Day 0, THR levels in Groups A and B were lower than Group C ($F = 1.64$, $P = 0.222$). At Day 31 THR levels in Groups B and C were significantly lower than Group A ($F = 1.11$, $P = 0.0351$), which did

not receive GonaCon™. At Day 61, THR levels in Groups B and C were equal and lower than Group A ($F = 2.22$, $P = 0.137$) (Table 2).

3.4. Rabies VNA titers

All dogs were reported to have been rabies vaccinated; dogs in Group B were not revaccinated against rabies. At Day 0, rabies VNA titers in Groups A and C were similar, but lower than Group B ($F = 2.46$, $P = 0.116$). At Day 31, rabies VNA titers in Group B were lower than Group A. Also, Group C titers were lower than Group B ($F = 3.01$, $P = 0.076$). At Day 61, rabies VNA titers in Groups B and C were lower than Group A ($F = 3.92$, $P = 0.040$) (Tables 3 and 4).

3.5. Anti-GnRH antibody titers

GonaCon™ was never used in these dogs before this study. At Day 0, no anti-GnRH immune response was detected. At Day 31, anti-GnRH titers among dogs in Groups B and C were similar, but higher than Group A ($F = 18.19$, $P = 0.0001$). The same results were observed on Day 61 ($F = 10.27$, $P = 0.001$) (Table 5).

3.6. Macro and microscopic observations

Six dogs in control Group A had no apparent abnormalities in their pituitary glands and skeletal musculature of their legs. Several atretic follicles were identified in the ovaries.

Four dogs in Group B showed slight diffuse edema and congestion in their pituitary glands. One sample had coagulative necrosis with basophilic cells and an increase in acidophilic cells. Three of

Table 3
Rabies virus neutralizing antibodies (VNA) by I.U. class for female dogs by Group and Day.

Group/Day	Group A			Group B			Group C		
	D0	D31	D61	D0	D31	D61	D0	D31	D61
Findings (IU/ml)									
<0.5	1	0	0	1	2	1	3	0	1
0.5–3.0	2	0	0	0	0	0	1	0	0
>3.0	3	6	6	6	5	6	3	7	6

Table 4
Mean and standard deviation for rabies virus neutralizing (VNA) levels for female dogs by Group and Day.

GROUP/DAY	Group A			Group B			Group C		
	D0	D31	D61	D0	D31	D61	D0	D31	D61
Statistical findings									
Mean	6.34	13.77	13.77	10.60	8.59	7.67	4.54	11.14	8.12
St dev	5.71	0.0	0.0	4.38	5.21	4.85	5.60	3.69	5.38

Table 5
Number of dogs with anti-GnRH antibodies by Group and Day.

GROUP/DAY	Group A			Group B			Group C		
	D0	D31	D61	D0	D31	D61	D0	D31	D61
Lab findings									
0	6	6	6	7	0	1	7	1	1
1/8	0	0	0	0	0	1	0	0	0
1/16	0	0	0	0	0	1	0	0	0
1/128	0	0	0	0	6	4	0	1	6
1/256	0	0	0	0	1	0	0	5	0

5 dogs showed no apparent changes in the skeletal muscles of their right and left hind legs. One of the other 2 dogs sampled showed moderate and focal chronic granulomatous myositis. The remaining dog showed diffuse coagulative necrosis.

Four of 6 dogs in Group C showed slight diffuse congestion in their pituitary glands. Three dogs also had diffuse edema. All samples showed coagulative necrosis with basophilic cells and an increase in acidophilic cells. Skeletal muscles of the right leg of 5 dogs had no apparent changes, but 2 of 5 left legs showed slight focal chronic granulomatous myositis. The other 3 had moderate to severe multifocal chronic myositis.

Overall, basophilia of the tunica albuginea, atresic follicles and reduction of the second, preatrium, and third follicles, were identified in ovary samples from 5 dogs in Group B and 6 dogs in Group C.

4. Discussion

Dogs in this study remained healthy, but serum analysis indicated chronic conditions and metabolic alterations associated with hepatic and renal functions. Additional analysis is necessary to determine if these conditions were a function of prior parasite loads and if they would have a measurable effect on the results. Because dogs in the study had previously received rabies vaccine, this value became the baseline for the control group. The administration of GonaCon™ vaccine resulted in a reduction of progesterone and high anti-GnRH antibody titers. No toxicity was reported in dogs treated with GonaCon™, but further study may be warranted to determine if the metabolic status of the dogs is an important criterion in their immune response. These results were similar to a previous study [5] in that the simultaneous administration of GonaCon™ and rabies vaccine in female dogs did not limit the immunogenicity of either vaccine.

During the 4 final days of this study, clinical evaluation detected apparent muscle atrophy at the injection site in 2 dogs from Group B and 5 dogs from Group C. Although systematic measurements were lacking over time, observations showed that the diameter of the muscles in the left leg was less than in the right leg. This apparent muscle atrophy near the GonaCon™ injection site was likely related to the chronic granulomatous myositis and diffuse coagulative necrosis reported in those animals after necropsy at D61. No limping, ulceration or paralysis was observed in these dogs. The number, type and intensity of adverse local reactions was lower than previously reported [22–24].

Two dogs in Group B, and 1 in Group C that began the study pregnant had puppies at D61. These dogs remained healthy throughout the study, providing supporting evidence that

GonaCon™ would not negatively affect existing pregnancies if this vaccine was used operationally.

The findings of this study are encouraging and advance our understanding of an improved formulation of GonaCon™. Additional studies to evaluate formulations that result in fewer local injection site effects while also reducing fecundity remain essential to move toward field use of GonaCon™ in Mexico during mass rabies vaccination campaigns. Moreover, additional monitoring of the effects of GonaCon™ on fertility through multiple breeding cycles (e.g., in owned dogs for 2 years) in the presence of reproductively competent male dogs remains critical to determine the complementary population management value of integrating this vaccine or perhaps other immunocontraceptives into dog rabies management. We contemplate future studies and offer encouragement to others to take similar initiatives in the interest of enhancing canine rabies management through the integration of less invasive, more efficient and effective dog population management methods in the future [25].

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