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Gonadotropin-releasing hormone immunization for the treatment of urethral sphincter mechanism incompetence in ovariectomized bitches

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ABSTRACT

We have investigated GnRH immunization for the treatment of urethral sphincter mechanism incompetence in ovariectomized bitches. It has been reported that decreasing LH secretion through the use of GnRH agonists temporarily restores continence in some bitches. Therefore, decreasing the circulating LH concentrations by immunizing against GnRH might temporarily maintain continence in incontinent dogs. Sixteen incontinent dogs given phenylpropanolamine (PPA) to control incontinence were recruited for this study. Eleven dogs were immunized against GnRH (novel treatment group) at week 0, and nine dogs were vaccinated again 4 weeks later. Five dogs (standard treatment group) were vaccinated with a placebo twice at 4-week intervals. PPA was discontinued in the novel treatment group 2 weeks after revaccination, and standard-treatment dogs were given PPA for the duration of the study. Blood samples were collected before each treatment and at 6, 8, 10, 12, 16, 20, and 24 weeks and owners recorded episodes of incontinence throughout the study. Ten of the eleven dogs in the novel treatment group experienced side effects as a result of vaccination; two of these dogs experienced more severe side effects after the first vaccination and were withdrawn from the study as a result. Of the nine dogs that completed the vaccination series, four dogs remained continent after PPA was discontinued. For these four dogs, there was no difference in incontinent episodes when they were given PPA versus treatment with the vaccine. All nine novel-treatment dogs developed a GnRH antibody titer and experienced a significant decrease in circulating LH concentrations. In conclusion, GnRH immunization was effective in maintaining continence in four of the nine incontinent ovariectomized dogs, and in these dogs, treatment with the vaccine was comparable with treatment with PPA.

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1. Introduction

The development of urethral sphincter mechanism incompetence (USMI) in female dogs is a prevalent sequelae after ovariectomy or ovariohysterectomy (hereafter referred to as ovariectomy). Incidence of USMI are reported to be between 5.7% and 20% in ovariectomized bitches [1,2], whereas only 0% to 1% of intact bitches

develop USMI [2]. After ovariectomy, urethral closure pressure decreases, even in bitches that remain continent [3]. However, urethral closure pressure is further reduced significantly in bitches with USMI [4].

Urethral closure pressure is normally maintained by sympathetic activation of α_1 -adrenoreceptors in the urethral smooth muscle [5]. Therefore, the most common current method of treating USMI is with α -adrenergic agonists, specifically phenylpropanolamine (PPA). Phenylpropanolamine mimics the effect of catecholamines by activating α_1 -adrenoreceptors in the urethral smooth muscle, effectively increasing urethral closure pressure and

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restoring continence. Unfortunately, PPA is not completely effective in the treatment of USMI [6,7]. In addition, PPA is not selective for α_1 -adrenoreceptors within the urinary tract. Undesired vascular smooth muscle contraction from PPA administration elsewhere in the body, including blood vessels, has been shown to cause hypertension in humans and mice [8]; this adverse effect has also been reported in dogs [9]. Other reported adverse effects of PPA in dogs include anorexia, emesis and weight loss, lethargy and behavior changes, and proteinuria [9]. Another clinical difficulty with the use of PPA is the 4-hour half-life [10] that requires dosing every 8 to 24 hours to maintain therapeutically effective urethral closure. This frequent administration can be frustrating for owners because treatment must be continued for the rest of the dog's life.

Ovariectomy results in elevated circulating concentrations of pituitary LH because there is no gonadal negative feedback. LH receptors are present throughout the canine urinary tract [11–13] and it has been postulated that elevated gonadotropins may contribute to the development of USMI [14]. Treatment of bitches with long-acting GnRH agonists downregulates LH secretion for prolonged time periods [15] and temporarily restores continence to incontinent bitches for varying durations, ranging from 50 to 738 days in one study [14] and 70 to 575 days in another [16]. Similar to PPA, GnRH agonists are not completely effective for the treatment of USMI [14,16]. However, unlike PPA, no adverse effects to GnRH agonists have been reported.

Unfortunately, GnRH agonist availability is limited in the United States. Although there are GnRH agonists available that are approved for the treatment of human diseases, such as prostate cancer, they are costly and not financially feasible for a pet owner to consider [17–19]. To date, deslorelin acetate (Suprelorin, Virbac Animal Health, Fort Worth, TX, USA) is the only GnRH agonist that has been developed for use in domestic animals [20]; however, it is currently available in the United States only for the treatment of adrenal disease in ferrets and extra-label use is explicitly prohibited [21].

There are other methods reported that temporarily decrease LH concentrations and therefore may also treat USMI, such as immunization against GnRH. Immunization against GnRH elicits the synthesis of GnRH-neutralizing antibodies, which prevent GnRH from binding to GnRH receptors and consequently prevent the synthesis of LH [22]. In 2004, a commercial GnRH vaccine was launched in the United States (Canine Gonadotropin Releasing Factor Immunotherapeutic; Pfizer Animal Health, Exton, PA, USA). This vaccine was labeled for the treatment of benign prostatic hyperplasia in intact male dogs with recommended revaccination every 6 months for effective treatment. It has also been shown to decrease testosterone concentrations in intact male dogs for approximately 20 weeks [23] and to safely terminate pregnancy in bitches [24].

The objectives of this study were to determine: (a) whether GnRH immunization will maintain continence in incontinent ovariectomized bitches; and (b) whether GnRH immunization controls USMI as effectively as PPA. It was hypothesized that GnRH immunization would effectively

maintain continence for a prolonged duration and that it would be as effective as PPA for the treatment of USMI.

2. Materials and methods

2.1. Animals and vaccination

Sixteen privately owned ovariectomized bitches were enrolled at Oregon State University's Veterinary Teaching Hospital under the oversight of Institutional Animal Care and Use Committee for this study. A diagnosis of incontinence after ovariectomy was confirmed using veterinary medical records. At the time the bitches that were recruited for the study were all being treated with PPA (Proin; PRN Pharmacal, Pensacola, FL, USA) to maintain continence. Dogs received varying doses of PPA as prescribed by their regular veterinarian; doses corresponding to each dog are summarized in Table 1. Clinical health was confirmed in all dogs by a complete blood count, biochemistry panel, urinalysis, and urine culture at the beginning and end of the study.

Novel-treatment dogs ($n = 11$) received 1 mL Canine Gonadotropin Releasing Factor Immunotherapeutic subcutaneously over the lateral thorax and were reimmunized 4 weeks later. Standard-treatment dogs ($n = 5$) received 1 mL saline over the lateral thorax and were treated again 4 weeks later. Animals were closely monitored by their owners for adverse reactions. One novel-treatment dog developed tachypnea for 24 hours after initial vaccination and another novel-treatment dog demonstrated impaired movement because of soreness for 1 week after initial vaccination. These two dogs did not receive a second vaccination and were excluded from further study.

2.2. Study design and sample collection

Venous blood samples were collected from standard- and novel-treatment dogs before each treatment (0 and 4 weeks) and again at 6, 8, 10, 12, 16, 20, and 24 weeks after initial vaccination. Blood samples were divided into Vacutainer clot tubes (02-685-A, Fisher Scientific Co., Waltham, MA, USA) to obtain serum and Vacutainer EDTA tubes (02-683-99 A, Fisher Scientific Co.) to obtain plasma. After centrifugation, serum and plasma were separated and frozen at -20°C until analysis.

Use of PPA was discontinued in novel-treatment dogs 2 weeks after the second vaccination, and standard-treatment dogs continued to receive PPA for the duration of the study. All novel-treatment dogs were not given PPA for at least 1 week after PPA was discontinued; when the dog became incontinent again, PPA administration was resumed.

Owners reported the frequency of incontinent episodes before any treatment for incontinence was initiated and they also recorded all episodes of incontinence for the duration of the study. For novel-treatment dogs that maintained continence after PPA discontinuation, comparisons were made between PPA treatment and treatment with the vaccine. The frequency of incontinent episodes on initial USMI diagnosis (before any treatment was initiated) during treatment with PPA (week 0 through week 6 of the

Table 1
Signalment of 16 ovariectomized dogs diagnosed with USMI.

Dog	Breed	Weight (kg)	Current age (y)	Age at ovariectomy (y)	Interval from ovariectomy to incontinence (y)	PPA dose (mg) and frequency	Study treatment	Treatment effect
1	Greyhound	26	9	1	5	25 BID	Vaccine	Continent
2	Golden Retriever	28	5	0.5	2.5	50 BID	Vaccine	Continent
3	Border Collie	18	11	1	0	50 SID	Vaccine	Incontinent
4	Pitbull	24	9	0.75	3.25	25 BID	Vaccine	Incontinent
5	Pitbull mix	20	1	0.25	0.25	50 BID	Vaccine	Incontinent
6	Doberman Mix	35	8	0.5	3.5	25 BID	Vaccine	Incontinent
7	Australian Cattle Dog	25	7	0.5	0	25 BID	Vaccine	Continent
8	Springer Spaniel	19	9	4.5	0.5	25 SID	Vaccine	Continent
9	Viszla	19	6	0.58	3.92	12.5 BID	Vaccine	Incontinent
10	Siberian Husky Mix	24	12	0.5	6.5	50 SID	PPA	Continent
11	Rottweiler Mix	34	8	0.5	3.5	50 BID	PPA	Continent
12	Labrador	27	6	0.5	2.5	25 SID	PPA	Continent
13	Weimaraner	27	9	4	3	25 BID	PPA	Continent
14	Weimaraner	29	6	2	3	25 BID	PPA	Continent
15	Australian Shepherd	25	6	0.5	3.5	25 SID	Vaccine	Rxn; Withdrawn
16	Australian Shepherd	20	9	0.5	2.5	25 SID	Vaccine	Rxn; Withdrawn
	Mean	25	7.56	1.13	2.71			
	Standard Deviation	5.09	2.61	1.29	1.81			
	Range	18–35	1–12	0.25–4.5	0–6.5			

Abbreviations: BID, twice daily; PPA, phenylpropanolamine; Rxn, reaction; SID, once daily; USMI, urethral sphincter mechanism incompetence.

study) and during treatment with the GnRH vaccine (week 7 through week 13 of the study) was compared, to determine the efficacy of each treatment type.

2.3. Sample assays

Serum samples were used to measure GnRH antibody titers, determined using ELISA using a technique modified from Elhay et al. [25]. Briefly, 96-well microtiter plates were coated with 100 μ L of 5 μ g/mL of LH-releasing hormone (71447-49-9, Sigma, St. Louis, MO, USA) in sodium bicarbonate buffer (pH 8.0) at 4 °C overnight. After incubation, plates were washed with PBS containing 0.05% Tween-20 (pH 8.0). Plates were then incubated for 1 hour at 20 °C with serum samples in duplicate diluted in a buffer containing 0.5% BSA (9048-46-8, Sigma) to yield final serum dilutions ranging from 1:8 to 1:1024. After tapping dry, antibodies were detected using horseradish peroxidase-protein G conjugate (10-1223, Invitrogen, Carlsbad, USA) diluted at 1:2000 in serum dilution buffer for 1 hour at 37 °C. After a final wash with PBS containing 0.05% Tween-20, horseradish peroxidase-protein G conjugate was visualized with 100 μ L of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) peroxidase substrate (50-66-01, KPL, Gaithersburg, MD, USA). Absorbance were read at 405 nm using a spectrophotometer (FLUOstar Omega, BMG Labtech Inc., San Francisco, CA, USA) and each serum sample was measured in duplicate. The cutoff for seropositivity, defined in this study as the upper limit of a 99% confidence interval above the mean negative control level, was calculated using the methods of Frey et al. [26]. Serological results were expressed as the reciprocal of the highest two-fold serial dilution above the calculated cutoff and linearized using a base-2 logarithmic scale.

Plasma samples were analyzed for LH concentrations in duplicate using an ELISA kit for canines (LH Detect;

Repropharm, Nouzilly, France), validated by Guérin et al. [27], and performed according to the manufacturer's instructions. The limit of detection was 0.12 ng/mL and the sensitivity was 0.2 ng/mL. The calculated coefficients of variation for intra-assay and interassay variability were 1% to 5% and 3% to 5%, depending on the LH concentration. Optical densities of the scale of standards and the samples were linearized using a base-e logarithmic scale and then back-transformed to determine the ng/mL LH concentration of each sample using a standard curve.

2.4. Statistical analysis

Serum GnRH antibody titers were compared between the novel treatment and standard treatment groups using Fisher's exact test (GraphPad QuickCalcs Software, Version 2013, La Jolla, CA, USA). Plasma LH concentrations were compared between the novel treatment and standard treatment groups as a repeated measure using PROC MIXED in SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA). Fixed effects in the repeated measure model were: whether the animal was vaccinated, time after first vaccination, and the interactions between vaccination and time. A first order heterogenous autoregressive variance-covariance structure was fitted for repeated measurements within animals. The frequency of incontinent episodes before initial treatment, treatment with PPA, and treatment with the vaccine were compared as a repeated measure using PROC MIXED in SAS. The fixed effect of the repeated measure model was the treatment type. Finally, LH concentrations and GnRH antibody titers were compared between novel-treatment dogs that maintained continence (MC) and novel-treatment dogs that did not maintain continence (DNMC) as a repeated measure using PROC MIXED in SAS. Fixed effects in the repeated measure model were: whether the animal maintained continence, time

after first vaccination, and the interactions between whether the animal maintained continence and time. A first order heterogenous autoregressive variance-covariance structure was fitted for repeated measurements within animals. Significance was defined as $P < 0.05$.

3. Results

The signalment, treatment, and result of treatment for all bitches are provided in Table 1. There were no differences between size, age, weight, age at ovariectomy, or interval between ovariectomy and incontinence in bitches from the novel treatment and standard treatment groups. There was no change in clinical health over the duration of the study determined according to the results of a complete blood count, biochemistry panel, urinalysis, and urine culture (data not shown).

As mentioned in the materials and methods section, two novel-treatment dogs were withdrawn from the study after the initial vaccination due to adverse reactions (tachypnea and prolonged soreness). Eight of the nine remaining novel-treatment dogs experienced minor side effects that included lethargy, swelling and/or soreness at the treatment site, and decreased appetite for approximately 24 hours after vaccination. Three dogs that experienced minor side effects following the initial vaccination were given diphenhydramine 1 mg/kg intramuscularly in conjunction with the booster vaccine. Owners of these dogs reported a decrease in adverse reactions following the second vaccination.

Four out of nine novel-treatment dogs remained continent after discontinuation of PPA. Of these dogs, one became incontinent again 14 weeks after PPA was discontinued (week 20) and received a third GnRH vaccination at that time. Incontinent episodes did not occur after the third vaccination and she remained continent through the end of the study. The other three dogs remained continent through the end of the study (24 weeks). All five standard-treatment dogs given PPA remained continent for the duration of the study. Of the four dogs in which the vaccine had a clinical effect, episodes of incontinence on diagnosis of USMI, during PPA treatment, and during vaccine treatment were compared. There were significantly less accidents when the dog was treated with PPA ($P = 0.02$) or the vaccine ($P = 0.01$) compared with before any treatment initiation. There was no significant difference in treatment between PPA and the vaccine (Fig. 1).

All dogs were seronegative for antibodies against GnRH before the first vaccination and all standard-treatment dogs remained seronegative for the duration of the study. All nine novel-treatment dogs developed a GnRH antibody titer after initial vaccination that peaked at week 6, and all but one dog still had an antibody titer at the end of the study (Fig. 2). When comparing GnRH antibody titers between MC dogs ($n = 4$) and DNMC dogs ($n = 5$), there was no significant difference in antibody titers at week 6, when PPA was discontinued ($P = 0.65$). However, DNMC dogs experienced a slower decline in antibody titer compared to MC dogs (Fig. 3).

At week 0, plasma LH concentration in all USMI dogs was 5.06 ± 1.99 ng/mL. After GnRH immunization, plasma LH concentration declined to basal levels in novel-

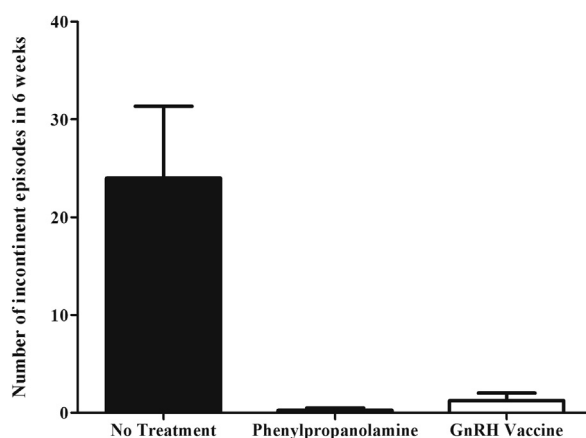


Fig. 1. Comparison of the number of incontinence episodes (\pm SD) that occurred in four out of nine novel-treatment dogs before any treatment was previously initiated, during treatment with phenylpropranolamine (PPA) from week 0 to week 6 of the study, and during treatment with the vaccine from week 7 to week 13 of the study. There were significantly fewer accidents when dogs were treated with PPA ($P = 0.02$) and the vaccine ($P = 0.01$) compared to before treatment initiation. There was no difference between treatment with PPA and treatment with the GnRH vaccine ($P = 0.88$).

treatment dogs (Fig. 4). Standard-treatment dogs maintained significantly higher LH concentrations (Fig. 4). When comparing LH concentrations between MC dogs ($n = 4$) and DNMC dogs ($n = 5$), there was no significant difference in LH concentrations between the two groups any time ($P = 0.67$) (Fig. 5).

4. Discussion

The development of USMI after ovariectomy in female dogs is a significant problem. Treatment with PPA is not completely effective, must be administered frequently to have a therapeutic effect, and may cause adverse side effects. Therefore, there is a need to find a safe therapy with a long-duration effect for the treatment of USMI. In this study, we reported that GnRH immunization was able to maintain continence in four out of nine incontinent

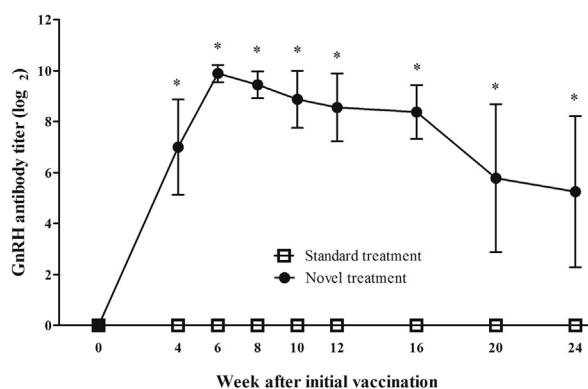


Fig. 2. Serum GnRH antibody titer (mean \pm SEM) in standard-treatment (open squares) and novel-treatment (filled circles) dogs before each treatment (0 and 4 weeks) and at 6, 8, 10, 12, 16, 20, and 24 weeks following initial treatment. * $P < 0.05$ compared to controls.

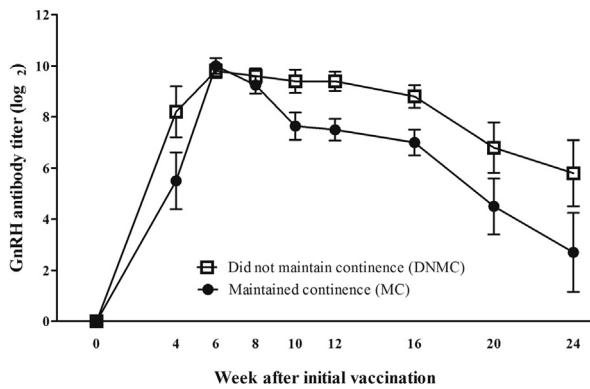


Fig. 3. Serum GnRH antibody titer (mean \pm SEM) in novel-treatment dogs that did not maintain continence (open squares) and dogs that did maintain continence (filled circles) before each treatment (0 and 4 weeks) and at 6, 8, 10, 12, 16, 20, and 24 weeks following initial treatment. There was no significant difference in titer at week 6, when dogs discontinued PPA ($P = 0.65$), but dogs that did not maintain continence experienced a slower decline in antibody titer.

ovariectomized dogs for 14 weeks or longer with an efficacy comparable to PPA in those dogs. However, side effects were observed as a result of vaccination in ten of the eleven vaccinated dogs.

Immunization against GnRH did not allow all novel-treatment dogs maintain continence in which is similar to the effects of GnRH agonists for the treatment of USMI. Reichler et al. [14,16] reported 41% to 54% efficacy for the treatment of USMI by reducing LH concentrations through the use of GnRH agonists. Reducing LH concentrations does not directly improve urethral pressure, but rather increases bladder threshold volume [28]. Although it is understood that the decrease in urethral pressure as a result of ovariectomy plays a role in the development of USMI [3], it has also been determined that ovariectomy results in changes in the bladder, specifically an increased collagen content and a reduced response of the detrusor muscle to muscarinic stimulation *in vitro* [29]. Therefore, it is possible that the changes in the bladder and the urethra might both

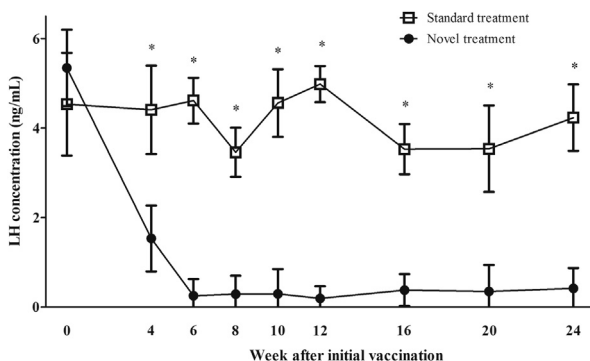


Fig. 4. Plasma LH concentrations (mean \pm SEM) in standard treatment (open squares) and novel treatment (filled circles) dogs before each treatment (0 and 4 weeks) and at 6, 8, 10, 12, 16, 20, and 24 weeks following initial treatment. There was a significant effect of the vaccine on LH concentrations ($P = 0.0004$). * $P < 0.05$ compared to controls.

contribute to the development of USMI. It is not known why increasing bladder threshold volume through the reduction of circulating LH is enough to restore continence in some, but not all, ovariectomized bitches. In some cases, an increase in bladder threshold volume might not be enough to compensate for decreased urethral pressure.

Although this assay has been used considerably in intact bitches, this is the first report utilizing the canine LH Detect ELISA to measure LH concentrations in ovariectomized bitches. Traditionally, LH is measured using RIA, which is a time-intensive and expensive process due to radiation use. Circulating LH concentrations measured in this study using ELISA of all USMI dogs at week 4 (average, 5.06 ng/mL; range, 1.99–8.39 ng/mL) were comparable to LH concentrations in 60 ovariectomized incontinent bitches measured using a canine-specific RIA (average 5.5 ng/mL, range 3–8 ng/mL) [30]. Circulating LH concentrations are affected by many factors, including body weight and age at ovariectomy [30]. Previously reported LH concentrations in ovariectomized bitches, some of small breeds (e.g., beagles) [15,31], did not take these factors into account and are therefore unfortunately not comparable with the results of this study.

All dogs with USMI recruited for this study were given varying doses of PPA (Table 1). Due to PPA's potential side effects, determining the lowest possible dose that still exerts a clinical effect is a common veterinary practice and results in varying doses between individuals. All dogs in this study were taking Proin chewable tablets, and the package label recommends a dose of 2 mg/kg twice daily (BID) [32]. However, studies have demonstrated successful treatment of USMI using doses of 1.5 mg/kg once daily (SID) [33] and 1 mg/kg three times daily (TID) [7]. Dogs in this study were healthy and continent when given their respective PPA doses and therefore, doses were not changed for the purposes of this study.

This study did not determine how long the GnRH vaccine had an effect on urinary incontinence because three of the four MC dogs were still continent when the study reached completion. Previous research on the effect of the GnRH vaccine in intact male dogs found that 20 weeks after initial vaccination, antibody titers were insignificant compared with prevaccination, and testicular volume was back to prevaccination measurements indicating the vaccine had lost its effect by that point [23]. Therefore, it was expected that the GnRH vaccine would also lose its effect approximately week 20 in the female dogs in this study. On the contrary, all novel-treatment dogs except one still had a GnRH antibody titer and all still had basal LH concentrations by week 24, indicating that the effect of the GnRH vaccine may be further prolonged in female compared with male animals. Regardless, reimmunization is inevitable. Based on the results of this study, reimmunization every 20 weeks is most likely necessary to allow animals sufficiently maintain continual continence, but further investigations are necessary to confirm this.

Interestingly, novel-treatment DNMC dogs experienced a slower decline in GnRH antibody titer than novel treatment MC dogs, though the difference was slight and may be due to the small sample sizes. Although responses to GnRH immunization are highly variable between animals [20], all

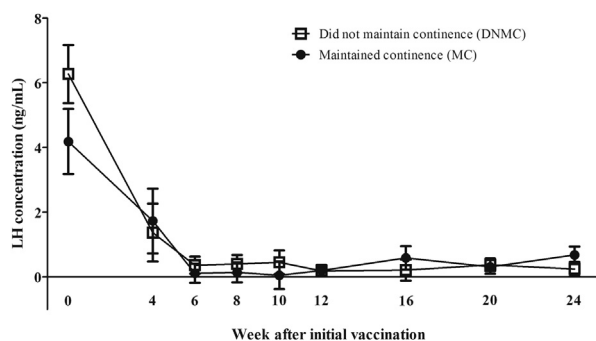


Fig. 5. Plasma LH concentrations (mean \pm SEM) in novel-treatment dogs that did not maintain continence (open squares) and dogs that did maintain continence (filled circles) before each treatment (0 and 4 weeks) and at 6, 8, 10, 12, 16, 20, and 24 weeks following initial treatment. There was no significant difference in LH concentrations between the two groups ($P = 0.67$).

novel-treatment dogs developed an antibody titer that was sufficient enough to decrease LH concentrations to basal levels for the duration of the study. Considering that there were no differences in LH concentrations between MC and DNMC dogs at any point of time, it is unlikely that the difference in GnRH antibody titer had contrasting effects on the two groups.

Nearly all novel-treatment dogs in this study experienced side effects. Although most side effects were minor and resolved without treatment after 24 hours, two dogs experienced adverse reactions that resulted in withdrawal from the study. Safety of the GnRH vaccine at 1 mL ($n = 237$) and 2 mL ($n = 24$) doses was reported in intact male dogs with no significant reactions or adverse events observed [34]. However, mild injection-site swelling occurred in 10% of the intact male dogs at the 1-mL dose and in 8.3% of the intact male dogs at the 2-mL dose. Minor side effects were also reported when the vaccine was used for pregnancy termination in bitches [24].

4.1. Conclusions

In conclusion, GnRH immunization was effective in maintaining continence in four out of nine incontinent ovariectomized dogs, and in these dogs, treatment with the vaccine was comparable to treatment with PPA. Because of low product sales, the GnRH vaccine investigated in this study is no longer commercially available. Efforts should be made to bring an available GnRH vaccine to market to provide another treatment option for USMI.

Competing interests

The authors have declared no conflicts of interest.

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