

Research Article

Effects of a Commercial Canine Gonadotropin Releasing Hormone Vaccination on Intact Male Llamas and Alpacas

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We have investigated the effect of immunization against gonadotropin releasing hormone (GnRH) using a commercial canine GnRH vaccine on testosterone concentration, testicular volume, testicular histology, and intermale behavior in intact male llamas and alpacas. Llamas ($n = 28$) and alpacas ($n = 20$) were either immunized (received 3 mL of vaccine given intramuscularly) or treated as controls (received 3 mL of sterile diluent given intramuscularly) at 0, 3, and 6 weeks. Blood samples and testicular volume measurements were taken at 0, 3, 6, 9, and 12 weeks. Owner surveys grading behavior at 0 and 12 weeks were received for 14 llamas. Two llamas at each time point undergoing the vaccination procedure were also castrated for testicular histological examination. Vaccinated animals elicited a GnRH antibody titer, and there was a significant decrease in testosterone concentration and testicular volume when compared with control animals. Intermale aggressive behavior was also significantly decreased in the surveyed llamas. However, histological examination revealed no significant changes. GnRH immunization using the canine GnRH vaccine may be an effective method for reducing intermale aggression in llamas and alpacas by decreasing circulating testosterone concentrations but cannot be recommended as an immunosterilant because of its lack of efficacy in interrupting spermatogenesis.

1. Introduction

The cohousing of intact male llamas and alpacas is a common farm arrangement for camelid owners in the United States, but keeping intact male camelids together frequently results in episodes of intermale aggression to assert territorial and social dominance. Aggressive behaviors include charging other males and forcing body contact, screeching, spitting, mounting, and biting [1]. If these behaviors are frequent, methods implemented by the owner to reduce aggression are necessary. Separating males from a herd can result in further behavioral problems because camelids are a highly social species [2]. Moving males around amongst intermale herds is not ideal either because hierarchal status must be reestablished with each change to the herd or reestablishing hierarchy results in male combat [1].

Castration is a recommended procedure utilized to reduce aggression [3, 4] and is frequently implemented to decrease intermale behavior in many species [2]. Surgical castration is expensive (several hundred dollars per animal) and irreversible. This may not be an ideal solution for owners that do not want the financial burden, want to preserve breeding stock, or want to avoid the risks of surgery. Therefore, there is a need for a cost-effective, reversible, and noninvasive means of reducing intermale aggression in camelids.

The hypothalamic-pituitary-gonadal axis has been a target for developing nonsurgical castration methods. Under normal conditions, hypothalamic gonadotropin releasing hormone (GnRH) stimulates the release of luteinizing hormone (LH) by binding to its receptor on the anterior pituitary. LH secretion is necessary for normal gonadal steroid hormone synthesis in males and females. Following GnRH

immunization, GnRH antibodies are produced that block GnRH binding to its receptor on the anterior pituitary. Without this GnRH-GnRH receptor binding, LH is not secreted, and this prevents synthesis of gonadal steroid hormones. In addition to significantly decreasing serum testosterone concentration, GnRH immunization also has been shown to result in testicular atrophy and disruption of normal spermatogenesis in multiple male species [5–8]. The latter effects result specifically from the withdrawal of testosterone as it is required for the maintenance of the seminiferous tubule epithelium in mammals [9].

Many mammalian species have been immunized against GnRH, including horses [5], rats [6], cats [7], sheep [8], swine [11], cattle [12], camels [13], and dogs [14]. To date, immunocastration with a GnRH vaccine is temporary because reproductive activity of immunized animals returns to normal once antibody titers drop off below an inhibitory threshold [15].

There is a GnRH vaccine commercially produced in the US (Canine Gonadotropin Releasing Factor Immunotherapeutic; Pfizer Animal Health, Exton, PA, USA) that is labeled for the treatment of benign prostatic hyperplasia in mature intact male dogs. Benign prostatic hyperplasia (BPH) is an androgen-dependent condition as the presence of androgens is essential for the development and maintenance of BPH [16]. Canine Gonadotropin Releasing Factor Immunotherapeutic is a nonsurgical approach for the treatment of BPH by eliciting GnRH antibodies that ultimately result in diminished testosterone concentration [17]. Earlier investigations in our laboratory using this vaccine have found decreased testosterone concentrations and testicular volume in intact male dogs [18] and decreased frequency of inappropriate urination in neutered male cats [19].

The objective of this study was to determine the humoral and hormonal effects of this canine GnRH vaccine in male llamas and alpacas. We hypothesized that immunizing camelids against GnRH would cause immunocastration (decreased testosterone concentrations, testicular atrophy, and diminished spermatozoa production) and decrease inter-male aggressive behavior.

2. Materials and Methods

2.1. Preliminary Safety Study. A preliminary safety study was conducted on three mature intact male alpacas to observe any adverse effects following administration of the Canine Gonadotropin Releasing Factor Immunotherapeutic vaccine (Pfizer Animal Health, Exton, PA, USA). One alpaca received a placebo (1.5 mL of a sterile diluent provided by the vaccine manufacturer administered intramuscularly into the left and right semimembranosus muscles for a total of 3 mL), one alpaca received a 1X dose (1.5 mL of the vaccine administered intramuscularly into the left and right semimembranosus muscles for a total of 3 mL), and one alpaca received a 2X dose (3 mL of the vaccine administered intramuscularly into the left and right semimembranosus muscles for a total of 6 mL) administered three times at three-week intervals (at week 0, week 3, and week 6). Serum chemistry panels were

determined prior to the first vaccine (week 0) and three weeks following administration of the last vaccine (week 9). Three weeks following the last injection, complete necropsies were performed on these animals to evaluate the potential toxicity of the vaccine.

2.2. Animals and Vaccination. Privately owned mature, intact male llamas ($n = 28$, age range 2–17 years) and alpacas ($n = 20$, age range 2–12 years) from Oregon were used in this study. The animals were housed in sex-aggregated groups and were cared for by their owners. Control animals received a placebo (1.5 mL of a sterile diluent provided by the vaccine manufacturer administered intramuscularly into the left and right semimembranosus muscle for a total of 3 mL; $n = 10$ llamas and 6 alpacas) and vaccinated animals received a 1X dose (1.5 mL of the Canine Gonadotropin Releasing Factor Immunotherapeutic vaccine administered intramuscularly into the left and right semimembranosus muscles for a total of 3 mL; $n = 18$ llamas and 14 alpacas). Treatments were administered three-times at three week intervals (at week 0, week 3, and week 6). Animals were closely monitored for adverse reactions for 48 hours following each vaccination. One alpaca developed pyrexia ($T = 103.4^{\circ}\text{F}$), lethargy, and bilateral hind limb lameness within 12 hours following the initial vaccination and was excluded from further vaccinations.

2.3. Behavioral Analysis. The owner of fourteen vaccinated llamas was asked to evaluate their social behavior before and after vaccination. These behaviors were classified as follows.

Score 1. (Calm). Quiet demeanor, does not start or participate in fights with other males, is not bothered by females within a visible range.

Score 2. (Moderate). Manageable demeanor, does not start fights with other males but will participate in them, is aware and pays attention to females within a visible range.

Score 3. (Aggressive). Dominant demeanor, starts and participates in fights with other males, focuses obsessively on females within a visible range.

2.4. Sample Collection, Scrotal Measurements, Determination of Antibody Titers, and Testosterone Concentrations. Jugular venous blood samples were collected from all animals prior to each injection (0, 3, and 6 weeks) and at 9 and 12 weeks following the initial treatment. All samples were allowed to clot; serum was prepared by centrifugation and stored at -20°C until assayed.

Left and right testes width and length were determined with digital calipers at the time of each blood collection. Each measurement was performed in triplicate by the same investigator to control for variation in measurement technique. Left and right testicular volume for each male were determined according to the formula $V = 4/3\pi ab^2$, where “ a ” is one-half the length and “ b ” is one-half the width [20]. A combined

TABLE 1: The criteria used for scoring changes within the seminiferous epithelium of vaccinated llamas at each time point, adapted from Yoshida et al. [10].

Score	Criteria
12	Many late spermatids or spermatozoa (≥ 10) present
11	Only a few late spermatids or spermatozoa (< 10) present
10	No spermatozoa and no late spermatids, but many round spermatids (≥ 10) present
9	No spermatozoa and no late spermatids, but only a few round spermatids (< 10) are present
8	No spermatozoa and no spermatids, but many secondary spermatocytes (≥ 10) are present
7	No spermatozoa and no spermatids, but only a few secondary spermatocytes (< 10) are present
6	No spermatozoa, no spermatids, no secondary spermatocytes, but many primary spermatocytes (≥ 10) are present
5	No spermatozoa, no spermatids, no secondary spermatocytes, but only a few primary spermatocytes (< 10) are present
4	No spermatozoa, no spermatids, no spermatocytes, many spermatogonia (> 10) are present
3	Only germ cells present are a few spermatogonia (< 10)
2	Absence of germ cells, but Sertoli cells are present
1	Total absence of cells in tubular section

total testes volume per animal was determined as the sum of the left and right testes volumes [20].

GnRH antibody titers were determined by ELISA using a technique derived from that previously described [21]. Briefly, 96-well microtiter plates were coated with 100 μ L of 2 μ g/mL of LH-RH (71447-49-9, Sigma, St. Louis, MO, USA) in sodium bicarbonate buffer (pH 8.0) and incubated at 4°C overnight. After incubation, plates were washed with phosphate-buffered saline containing 0.05% Tween-20 (TPBS) (pH 8.0) and were blocked with StabilCoat (SC05-0100, SurModics, Eden Prairie, MN, USA). After blocking and washing with TPBS, plates were incubated for 1 hour at 20°C with serum samples diluted in a buffer containing 0.5% bovine serum albumin (9048-46-8, Sigma, St. Louis, MO, USA) to yield final serum dilutions ranging from 1:16 to 1:2048. After another wash with TBPS, antibodies were detected using horseradish peroxidase protein G conjugate (HRP) (10-1223, Invitrogen, Camarillo) diluted at 1:2000 in serum dilution buffer for 1 hour at 37°C. After a final wash with TPBS, HRP was visualized with 100 μ L of ABTS peroxidase substrate (50-66-01, KPL, Gaithersburg, MD, USA). Absorbances 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 95% confidence interval above the mean negative control level, was calculated using the methods of Frey et al. [22]. Serological results are expressed as the reciprocal of the highest twofold serial dilution above the calculated cutoff and normalized using a base-2 logarithmic scale.

Serum samples were also analyzed for testosterone concentration at the Animal Health Diagnostic Center at Cornell University using a Coat-A-Count Total Testosterone radioimmunoassay (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) and performed according to the manufacturer's instructions.

2.5. Castration and Histological Evaluation. Two llamas at each time point (weeks 0, 3, 6, 9, and 12) undergoing the

vaccination procedure outlined previously were castrated using a routine scrotal castration technique. Therefore, the two llamas castrated at week 0 received no vaccinations, the two at week 3 received one vaccination, the two at week 6 received two vaccinations, and the two at weeks 9 and 12 received three vaccinations. Briefly, the llamas were generally anesthetized with a 0.3 mL of a mixture of ketamine (83 mg/mL), xylazine (8 mg/mL), and butorphanol (0.8 mg/mL) [23]. A 2 cm incision was made on either side and parallel to the median raphe along the most ventral aspect of the scrotum [24]. Each testicle was removed and excised using an emasculator. The skin incisions healed by second intention after castration.

Each testis was hemisectioned and fixed, paraffin-embedded, cut into 6 μ m sections, and stained with hematoxylin and eosin. Histological evaluation was performed by a veterinary pathologist blinded to the treatment groups. The evaluation included assessment of Leydig cell density and morphology along with the proliferation and maturation of spermatocytes/spermatids. Leydig density was scored on a 0–3 scale: 0 = no cells present; 1 = scattered/few cells present; 2 = moderate number of cells present; 3 = densely packed within the interstitium. Leydig morphology was scored on a 0–3 scale: 0 = normal; 1 = mild changes; 2 = moderate changes; 3 = severe changes. Seminiferous tubules were studied using the Yoshida scoring method, which gives a score of 1–12 according to the presence or absence of germ cells (see Table 1) [10].

2.6. Statistical Analysis. Analyses were carried out using SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA). Antibody titers of GnRH were log-transformed to the base 2 to achieve normalization, testosterone concentrations and testicular volumes were analyzed as percent change from baseline (0 weeks), and these data were analyzed as a repeated measure in time design in PROC MIXED. Fixed effects in the model were vaccination (yes, no), time after first vaccination (3, 6, 9, and 12 weeks), species (llama, alpaca), and the interactions between vaccination and time and between vaccination and

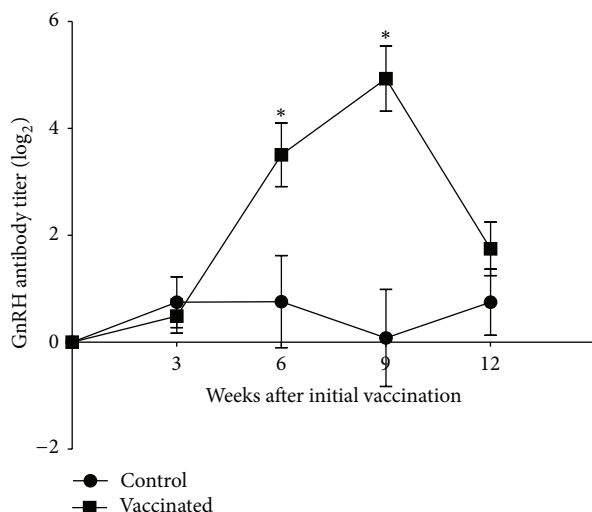


FIGURE 1: Serum GnRH antibody titer (\pm SEM) in control (●) and vaccinated (■) animals prior to each injection (0, 3, and 6 weeks) and at weeks 9 and 12 following initial treatment. * $P < 0.05$ compared to controls.

species. A first order heterogeneous autoregressive variance-covariance structure was fitted for repeated measurements within animals. Age of animal was not included as fixed effect in the model because the effect was not significant. Behavior scores before and after vaccination were compared using the nonparametric sign test. Histology was evaluated using PROC GLIMMIX for generalized linear mixed models. Significance for all data was defined at $P < 0.05$.

3. Results

Results of the preliminary safety study showed no evidence of biochemical analysis or necropsy of any toxicological effect of the vaccine. There were no significant changes in the serum biochemistry from the beginning to the end of the safety study. The postmortem examinations found the animals to be in good body condition prior to euthanasia. Gross and histopathologic evaluations of all the organs examined were unremarkable with the exception of evidence of intestinal parasitic migration (e.g., fibrous scars present on the diaphragmatic capsular surface of the liver) in the control and in the 2X dose alpacas.

Excluding the alpaca that developed pyrexia, lethargy, and bilateral hind limb lameness following the initial vaccination, mild injection site reactions were observed in six vaccinated llamas and no alpacas. Five llamas experienced swelling at the injection site after the first injection but only one of those five experienced swelling at the injection site after the second injection as well. None of the five experienced a reaction to the third injection. The sixth llama experienced hind limb lameness after the first injection but did not experience a reaction to the second or third injection.

All animals were seronegative for antibodies against GnRH prior to the first vaccination, and there was no significant effect of species on GnRH antibody titer over time

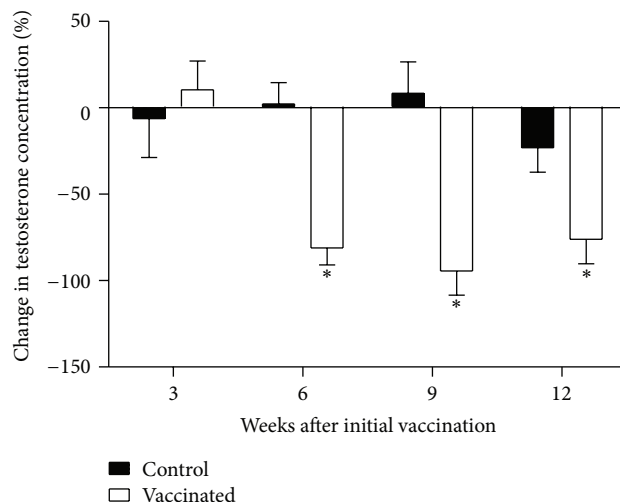


FIGURE 2: Change in testosterone concentration (%) in control (■) and vaccinated (□) animals prior to each injection (0, 3, and 6 weeks) and at weeks 9 and 12 following initial treatment. * $P < 0.05$ compared to controls.

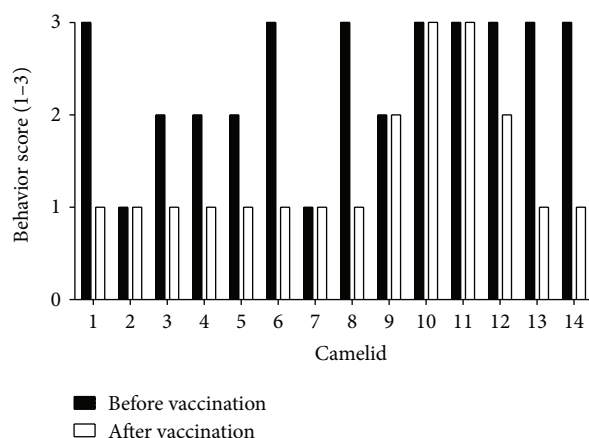


FIGURE 3: Change in behavior score before initial vaccination (■) and after completion of the vaccination series (□) in 14 llamas. Score 1 = calm, score 2 = moderate, and score 3 = aggressive.

($P = 0.1$). Compared to control animals, antibody titers of vaccinated animals were significantly greater at week 6 ($P = 0.01$) and week 9 ($P < 0.0001$) (Figure 1). Antibody titers of vaccinated animals peaked at 9 weeks after vaccination.

There was no significant effect of species on the change in testosterone over time ($P = 1.0$). Compared to controls, testosterone concentrations in vaccinated animals were significantly decreased at week 6 ($P < 0.0001$), week 9 ($P < 0.0001$), and week 12 ($P = 0.01$) (Figure 2). Furthermore, of the fourteen vaccinated llamas evaluated behaviorally, there was a significant decrease in behavior score ($P = 0.002$) following the third vaccination. Of the 8 males identified as being aggressive at the time of vaccination, 5 (62.5%) experienced a marked decrease in aggression, whereas a minimal decrease was seen in 1 male (12.5%) and no change was seen in 2 males (25%) (Figure 3).

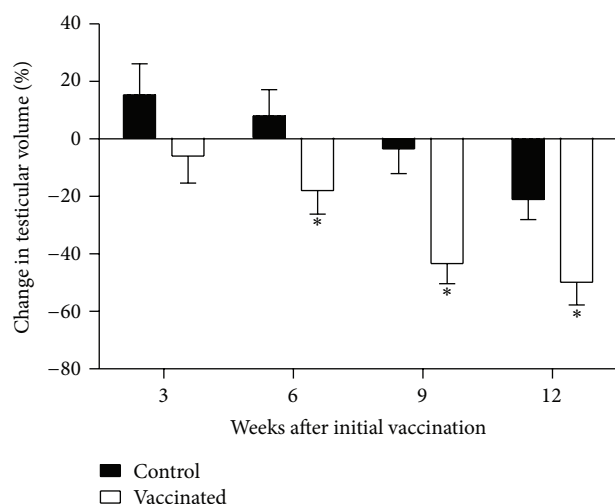


FIGURE 4: Change in testicular volume (%) in control (■) and vaccinated (□) animals prior to each injection (0, 3, and 6 weeks) and at weeks 9 and 12 following initial treatment. * $P < 0.05$ compared to controls.

There was no significant effect of species on the change in testicular volume over time ($P = 0.6$). Compared to controls, testicular volumes in vaccinated animals were significantly decreased at week 6 ($P = 0.04$), week 9 ($P = 0.002$), and week 12 ($P = 0.01$) (Figure 4). Unexpectedly, there was no effect of GnRH immunization on spermatogenesis based on seminiferous tubule scoring ($P = 0.6$), Leydig cell morphology ($P = 0.2$), or Leydig cell density ($P = 0.6$) (Figure 5).

4. Discussion

Immunization against GnRH has been used as an alternative method of castration in various species [5–8, 11–14]. Immunocastration is implemented in order to prevent undesirable effects of androgens, including aggressive behavior. We hypothesized that immunization against GnRH in llamas and alpacas would result in a decrease in serum testosterone concentration, testicular volume, sperm production, and intermale aggression. This is the first study to investigate an alternative means of castrating llamas and alpacas through GnRH immunization.

This study demonstrates that camelids are capable of generating GnRH antibodies following immunization against GnRH, which was correlated with a significant decrease in testosterone concentrations and testicular volume. The average testosterone concentration of vaccinated camelids was <1000 pg/mL, which was below normal parameters reported for adult camelids [25]. It is of interest to note that the control animals showed an apparent fluctuation in testosterone concentration during the study period (Figure 2), which had been previously observed by Bravo et al. [26] in male llamas in the United States from 30 to 50 months of age. Furthermore, in a study conducted in male vicuñas

where blood samples were taken every 15 minutes for 4–8 hours, testosterone profiles showed high variation among animals and fluctuation throughout the sampling period [27].

Not all participating owners submitted behavior evaluations before and after treatment. However, there was a significant decrease in intermale aggressive behavior in the 14 surveyed llamas, which was correlated with an elevated GnRH antibody titer and reduced testosterone concentration. Furthermore, it is of interest to note that behavior scoring was also performed on two control llamas that were cohoused with the vaccinated llamas. The owner noted that while the behavior of one of the control llamas did not change, the behavior of the other control animal became more aggressive towards the vaccinated males it was cohoused with. This may be the result of the decreased aggressive behavior displayed by the vaccinated males, allowing the control llama to assume a higher hierarchal status. However, further study with a larger subset of animals is necessary to elucidate whether partially vaccinating a herd of co-housed males can alter established herd hierarchies.

While the change in testicular volume decreased significantly more in vaccinated animals compared to controls, there was also an apparent decrease in testicular volume in the controls over time (Figure 3). This study took place during the summer in the USA, so it is possible that season could have affected testicular volume. In male vicuñas, seasonal variation was found to affect testes size [27]. However, the mechanism regarding seasonality in male camelids is poorly studied [25] and further research on seasonality in male camelids with respect to geographic location and management practices is necessary to determine whether season can affect testicular volume.

Even though there was a significant change in testicular volume in vaccinated animals compared to controls, there was no significant difference in seminiferous tubule score, Leydig cell density, or Leydig cell morphology between the different groups of castrated llamas. It is generally accepted that testosterone is required to maintain spermatogenesis in the adult. However, normal spermatogenesis can be maintained despite up to 80% reduction in intratesticular testosterone concentrations in rats [28, 29]. It is also important to note that intratesticular testosterone concentration is much higher (approximately 50 ng/mL) compared to circulating testosterone concentration (1–2 ng/mL) [30]. This suggests that spermatogenesis in camelids could be maintained despite circulating testosterone concentration below the limits of detection, which is what we observed in the current study. Further study is needed in camelids to compare circulating testosterone concentration to intratesticular testosterone concentration in GnRH vaccinated males.

In conclusion, GnRH immunization using the canine GnRH vaccine may be an effective method for temporarily reducing intermale aggression amongst co-housed male camelids by decreasing circulating testosterone concentrations. However, further investigation is needed before GnRH immunization using the canine GnRH vaccine can be recommended as an immunosterilant because of the lack of efficacy

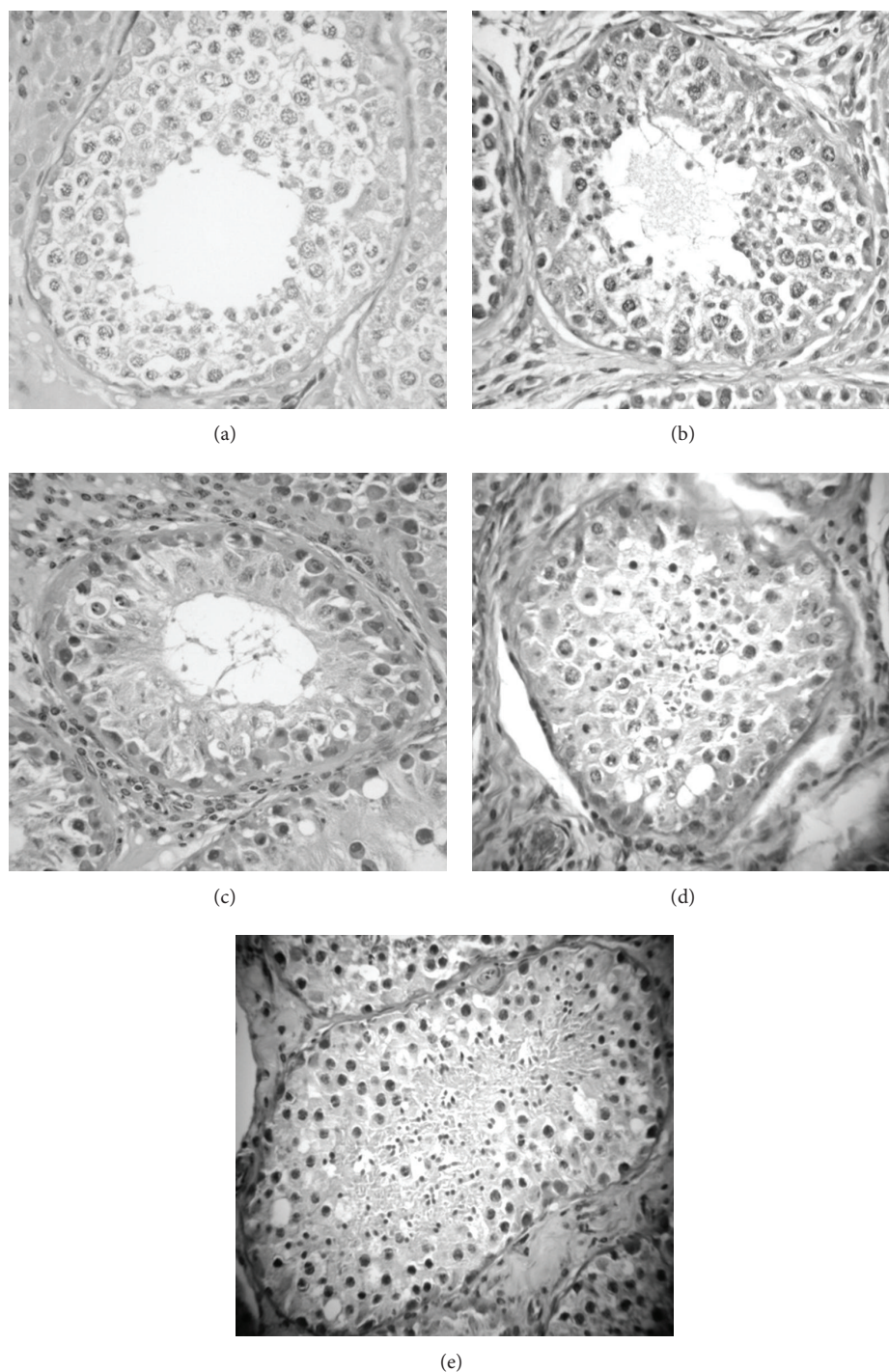


FIGURE 5: Cross-sections of testes at 20x magnification from a nonvaccinated llama (a) and vaccinated llamas at week 3 (b), 6 (c), 9 (d), and 12 (e) following initial treatment. There was no effect of GnRH immunization on spermatogenesis at any time point based on seminiferous tubule scoring ($P = 0.6347$) on Leydig cell morphology ($P = 0.1987$) or density ($P = 0.6256$).

in interrupting spermatogenesis that was observed in this study.

Conflict of Interests

The authors have declared no conflict of interests.

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