Effect of immunization with bovine luteinizing hormone receptor on ovarian function in cats

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Objective—To determine the effect of immunization with bovine luteinizing hormone receptor (LH-R) on ovarian function of cats.

Animals—9 adult female domestic cats.

Procedure—7 cats were immunized with 0.5 mg of LH-R encapsulated in a silastic subdermal implant (3 X 10 mm); 2 served as control cats. Receptors had 80% specific binding to 125I-human chorionic gonadotropin with a binding capacity of 2,682 pM/mg. Cats received booster injections of LH-R. Cats were induced to ovulate with luteinizing hormone (LH) releasing hormone on day 345. Samples of venous blood and vaginal cells were collected through day 395. Observation of estrus behavior continued until day 516. Serum concentrations of estradiol, progesterone, thyroid gland hormones, LH, and LH-R antibody were determined.

Results—LH-R antibody was detected in the sera of immunized cats within 21 days after implantation. Detection of LH-R antibody was associated with suppression of serum progesterone to <0.5 ng/mL during the study period, compared with concentrations of 5 to 10 ng/mL in control cats. Immunized cats did not display signs of estrus. Release of LH after administration of LH-releasing hormone indicated an intact hypothalamic-pituitary axis but poor corpus luteum function. Serum estradiol concentrations remained between 30 to 40 pg/mL in immunized and control cats. With the decrease antibody titer, hormone concentrations returned to a pattern consistent with that during fertility.

Conclusions and Clinical Relevance—Active immunization with LH-R suppressed corpus luteum function in cats. The effect was reversible. An LH-R-based antifertility vaccine may have clinical application in other vertebrates. (Am J Vet Res 2003;64:292–298)
Preparation of LH-R—Plasma membranes from bovine luteal cells, which contain LH-R, were solubilized in 1% Triton X-100 and purified as described elsewhere. Triton X-100 was removed from the purified receptors by the use of a commercial product in accordance with the manufacturer’s instructions. The concentration of Triton X-100 was determined by using the method of Garewal. Starting with 100 g of bovine corpora lutea protein, we obtained 14.6 mg of purified receptor. The purified receptor had 80% specific binding of bovine corpora lutea protein, we obtained 14.6 mg of purified receptor. The purified receptor had 80% specific binding of bovine corpora lutea protein, we obtained 14.6 mg of purified receptor.

Adjuvant—We used N-acetylgalcosaminyl-(β1-4) N-acetylmuaramyl-l-alanyl-l-isoglutamine (GMDP), a potent stimulant of the immune system, as an adjuvant. The adjuvant was prepared from a subunit of mycobacterium cell wall. The GMDP activates macrophages and B and T lymphocytes and causes release of interleukins, thereby promoting cellular immunity.

LH-R implants—Silastic tubing (3 X 10 mm) with an inside volume of approximately 100 µL was microperforated by use of a 25-gauge heated needle. In our experience, these microperforations will allow slow diffusion of LH-R from inside the silastic tubes to the surrounding environment. Silastic tubes were washed with 70% ethanol, dried under sterile conditions, and placed in an upright position in a sterile vial. Purified receptor was solubilized in 0.2% Triton X-100, emulsified with adjuvant, and sterilized by ultrafiltration through a 0.45-µm filter. The LH-R solution contained 14.4 mg of protein/mL; this solution was poured into each sterile vial until all the microperforated silastic tubes were submerged and, thus, filled with the LH-R solution. Air bubbles trapped within the silastic tubes were removed by suction and use of a sterile syringe and needle. Silastic tubes filled with the receptor solution (ie, implants) were lyophilized and stored individually in sterile vials at 4°C in a desiccator until implanted in the cats. Adjuvant was used at a rate of 5 µg/kg of body weight in a ratio of 0.5 mg of LH-R to 1.44 mg of adjuvant (total of 100 µL of solution/implant). Thus, each implant contained 0.5 mg of LH-R protein. Sterility of the purified LH-R and the LH-R implants was achieved by use of UV radiation, and implants were confirmed to be free of bacterial and viral contamination by examination at a laboratory.

Surgical insertion of implants—Implantation was performed in cats anesthetized by IM injection of 6 to 10 mg of tiletamine-zolazepam/kg. Microchips were implanted simultaneously with the LH-R implants; the microchips were used to identify the cats.

Skin between the scapulas was shaved and prepared by cleaning with an iodine-based solution and alcohol. A sterile drape was placed over the cats back, exposing only the shaved area. A 3-mm incision was made through the skin. A stylet with the canula of a trocar containing the microchip was introduced through the incision. The canula was advanced subcutaneously for a distance of 3 cm. The stylet was withdrawn from the canula, leaving the microchip beneath the skin. By use of sterile forceps, the LH-R implant was inserted to a depth of approximately 2 cm through the groove created by the trocar. The incision was closed by use of 2-0 silk sutures and surgical staples.

Experimental design—The study began in March. Seven cats (treatment group) were each implanted with an implant that contained LH-R, whereas 2 cats (control group) were each implanted with an implant that contained only adjuvant and saline (0.9% NaCl) solution (day of implantation was designated as day 0). During the postimplantation period, aliquots of 0.1 mg of purified LH-R, emulsified in adjuvant were injected IM into experimental cats on days 98, 139, 160, and 193 to sustain immunogenic stimulation and maintain antibody titers. Cats in the control group were injected at the same times with adjuvant and saline solution.

Collection of blood samples—Prior to implantation on day 0 and at intervals of 14 to 21 days thereafter through day 395, cats were sedated, and blood samples and samples of vaginal cells were obtained. Blood samples (5 mL) were collected by venipuncture of a cephalic or jugular vein. Serum was separated by use of centrifugation and stored at –20°C until analyzed.

Vaginal cytologic examinations—Vaginal cells were obtained on the same days as the collection of blood samples. Vaginal cytologic examinations were performed in a routine manner. Vaginal cells were obtained by passing a cotton-tipped swab moistened with sterile saline solution into the vagina. The swab was directed cranio-caudally during entry into the vagina and was rubbed against the cranial vaginal wall. The vestibule and clitoral fossa were avoided during the procedure, because superficial cells from those areas could have altered the cytologic interpretation. Cells were transferred to a glass slide as a smear by gentle rotation of the swab. Slides were air dried, stained with Wright stain, and examined by use of light microscopy. Cell types were identified to assign the stage of the estrous cycle. Collection of vaginal cells was sometimes compromised to avoid undue discomfort to the cats.

Assessment of stage of estrous cycle—Signs of estrus behavior were monitored and recorded prior to immunization and throughout the course of the study. From day 395 until 516, cats were housed at the College of Veterinary Medicine, Cornell University, and clinician at that facility monitored estrus behavior during that period. Hormone concentrations and daily estrus behavior were also considered when assigning the stage of the estrous cycle.

Ovulation induction—Cats immunized with LH-R appeared to be infertile, as indicated by suppression of P₄ concentrations and anestrus indicated by results of vaginal cytologic examination. To confirm the apparent state of infertility, all cats were administered LH-releasing hormone on day 345. Injections of 50 µg of LH-releasing hormone were administered IM at 0 and 6 hours. Blood was collected from each cat before and 12 hours after the first injection and on day 8 after LH-releasing hormone injections. Serum was harvested, and concentrations of LH, E₂, and P₄ were determined in samples obtained 12 hours and 8 days after the first LH-releasing hormone injection.

Determination of hormone concentrations—Serum concentrations of E₂, P₄, and LH were determined by use of radioimmunoassays. Sensitivity of the radioimmunoassays for E₂ and P₄ were 0.6 and 0.19 pg/mL, respectively, with a correlation coefficient of 0.9 and 0.955, respectively. Intra- and interassay coefficients of variation of the assays were 0.33 and 0.17 for E₂ and 0.03 and 0.16 for P₄, which were similar to values reported elsewhere.

Serum LH concentrations were determined by use of a heterologous radioimmunoassay that used anti-rabbit LH

Animal Care International. Cats were provided ad libitum access to water and a commercially available food. There were no male cats in the vicinity. The experimental protocol was approved by an institutional animal care and use committee.

A baseline CBC count and serum biochemical analysis were performed. On the basis of results of those tests, the cats were determined to be in a general state of good health. The cats also had negative results when tested for endoparasites, heartworms, Salmonella spp, Shigella spp, feline immunodeficiency virus, and FeLV.
antibody. Sensitivity of the LH assay was 0.66 ng/mL. Mean recovery of low, medium, and high concentrations of added LH was 96.2, 95.6, and 89.3%, respectively. Typical diestrous concentrations of serum LH in cats range from 0.2 to 1.8 ng/mL with a mean of 1.0 ng/mL.

Concentrations of thyroid gland hormones (namely triiodothyronine [T3], bound thyroxine [T4], and free T4) were measured with a chemiluminescent immunoassay that used acridinium ester-labeled antibody. Concentrations were determined in accordance with the manufacturer’s instructions. Thyroid-stimulating hormone was not measured, because an assay was not available.

**Determination of LH-R antibody concentrations**—Serum LH-R antibody titers were determined by use of a modification of a radioreceptor assay.15 Iodinated hCG (specific activity, 50 µCi/µg) was obtained from a commercial vendor. The 125I-hCG was purified by gel-filtration through a column of agarose to remove damaged hCG and free 125I. The column was equilibrated and eluted with 0.05M Tris-Cl buffer (pH, 7.5) that contained 1% bovine serum albumin. Fractions (0.5 mL) were collected, and 10-µL aliquots of each fraction were counted in a gamma counter. Fractions in the first eluted peak that had high specific activity were pooled to yield purified 125I-hCG, which was diluted with Tris-Cl buffer to yield a final product with approximately 30,000 counts per minute (CPM)/100 µL.

Aliquots (50 µL) of serum samples obtained from the cats were tested for inhibition of specific binding to the purified fraction of the 125I-hCG plasma membrane receptor obtained from bovine corpora lutea. Fifty-microliter aliquots of plasma membrane fraction containing 500 µg of protein were placed in 10 × 75-mm disposable plastic tubes. We then added 100 µL of 125I-hCG containing 30,000 CPM, with and without 1 µg of unlabeled hCG in 10 µL of Tris-Cl buffer, which was followed by the addition of a sufficient quantity of Tris-Cl buffer to achieve a final concentration of 500 µL; tubes were incubated at 37°C for 1 hour or 4°C for 16 hours. A γ-globulin fraction of a monoclonal antibody to LH-R that had a mean ± SEM inhibition of 74.85 ± 4.3% for 125I-hCG binding to the bovine luteal plasma membrane was dissolved in normal cat serum and used as an internal-control sample.

After incubation, tubes were centrifuged (3,228 × g) in a refrigerated centrifuge. Supernatant was discarded, and pellets were counted in the gamma counter. Feline serum and the γ-globulin fraction of LH-R antibody generated in rabbits did not bind 125I-hCG. Nonspecific serum effects were subtracted from the antibody titer in the serum samples obtained from the cats. Antibody titers to LH-R were calculated as

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the percentage inhibition of specific binding of \(^{125}\)I-hCG to the receptor.

Analysis of data—Results of ovulation induction after administration of LH-releasing hormone, serum concentrations of LH, \(E_2\), and \(P_4\), results of vaginal cytologic examination, and assessment of estrus and the stages of the estrous cycle in immunized and control cats were determined. Values for those variables were correlated with LH-R antibody titers. The \(P\) values were derived by use of \(t\)-tests to determine significant differences. Values of \(P < 0.05\) were considered significant.

Results

Serum concentrations of \(E_2\) and \(P_4\), as well as results of vaginal cytologic examinations of the 7 cats immunized against LH-R were recorded (Fig 1). Similar data were recorded for the 2 control cats (Fig 2). Results for the vaginal cytologic examinations represent the best consensus of all investigators. Because of variation in the antibody response among cats, results of vaginal cytologic examinations may not have reflected exactly the expected vaginal cells corresponding to normal ovarian function. Prior to implantation, all cats had normal estrus behavior and probably had at least 1 estrous cycle. Circulating concentrations of LH-R antibody were detected on day 20 after implantation in the immunized cats; values ranged from 50 to 70% inhibition of \(^{125}\)I-hCG binding to the receptor.

The \(E_2\) concentrations in serum samples of immunized cats revealed a pattern that could not be compared precisely with that of control cats, which was the result of the fact that \(E_2\) concentrations were measured only every 14 to 21 days because of the limitations of the experimental protocol. However, analysis revealed that serum \(E_2\) concentrations in immunized cats (range, 20 to 40 pg/mL; Fig 3) were not significantly different from concentrations in the 2 control cats (range, 10 to 30 pg/mL; Fig 2).

Coincident with the appearance of LH-R antibody in the immunized cats, serum \(P_4\) concentrations were suppressed and remained suppressed throughout the entire period during which cats were receiving booster injections. The cats did not display signs of estrus during this period. During the observation period between days 395 and 516, clinicians at the College of Veterinary Medicine, Cornell University determined that the cats displayed estrus (as indicated by vocalization and physical behavior), suggesting a return to estrus during that time frame. The cats were not exposed to male cats during this period. In the control cats, increases in serum \(E_2\) and \(P_4\) concentrations were accompanied by signs of estrus.

Immunized and control cats were induced to ovulate on day 345 by administration of LH-releasing hormone (Table 1). Serum samples collected 12 hours after administration of the first injection of LH-releasing hormone revealed a mean LH surge of 2 ng/mL for the immunized cats and 3 ng/mL for the control cats.
control cats, compared with LH concentrations of 0.5 and 0.26 ng/mL, respectively, before injection, indicating that the pituitary reserves of LH were intact. Serum P₄ concentrations in control and immunized cats were negligible at 12 hours after the first injection of LH-releasing hormone. On day 8 after injection of LH-releasing hormone, mean serum P₄ concentrations increased to 2.74 ng/mL in control cats, but little P₄ was detected in the sera of the immunized cats, possibly indicating poor corpus luteum function. However, we did not obtain biopsy specimens of corpora lutea for histologic examination to confirm this assumption.

Mean ± SEM concentrations of T₃ in the sera of all cats prior to implantation (23 ± 0.04 ng/dL) did not differ significantly (P = 0.8) from concentrations in control cats that received implants without LH-R (24 ± 0.05 ng/dL). However, concentrations in the immunized cats (34 ± 0.01 ng/dL) differed significantly from values for all cats prior to implantation (P = 0.02) and from values for cats that received implants without LH-R (P < 0.05). Mean concentrations of T₃ did not differ significantly (P = 0.2) between cats prior to implantation (0.01 ± 0.002 µg/dL) and control cats after implantation (0.07 ± 0.04 µg/dL). However, T₄ concentrations in immunized cats (0.3 ± 0.01 µg/dL) were significantly higher, compared with values for all cats prior to immunization (P = 0.01) and with values for cats that received implants without LH-R (P < 0.05). This could have been potentially attributable to an increase in thyroid-binding protein, because mean free T₄ concentrations did not differ significantly for cats prior to implantation (1.45 ± 0.01 ng/mL), control cats that received implants without LH-R (1.8 ± 0.16 ng/mL), and immunized cats (1.65 ± 0.01 ng/mL). Furthermore, it is recognized that free T₄ concentrations are clinically more important than bound T₄ concentrations.¹⁶ We did not detect clinical signs suggestive of a thyroid gland disorder. In addition, we did not observe other local or systemic adverse effects.
Table 1—Mean ± SEM concentrations of various hormones after injection of 2 doses of luteinizing hormone (LH)-releasing hormone on day 345 after implantation of an implant containing bovine LH-receptor in 7 cats (immunized cats) or implantation of a sham implant in 2 cats (control cats).

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Cats</th>
<th>0 hours</th>
<th>12 hours</th>
<th>8 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (ng/mL)</td>
<td>Immunized</td>
<td>0.50 ± 0.50</td>
<td>2.00 ± 0.30</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Control*</td>
<td>0.26 (0.21–0.31)</td>
<td>3.00 (2.87–3.13)</td>
<td>ND</td>
</tr>
<tr>
<td>Progesterone (ng/mL)</td>
<td>Immunized</td>
<td>0.21 ± 0.15</td>
<td>ND</td>
<td>Trace to 0.23</td>
</tr>
<tr>
<td></td>
<td>Control*</td>
<td>0.38 (0.20–0.56)</td>
<td>ND</td>
<td>2.74 (2.72–2.76)</td>
</tr>
<tr>
<td>Estradiol-17β (pg/mL)</td>
<td>Immunized</td>
<td>21.50 ± 11.36</td>
<td>ND</td>
<td>13.08 ± 4.00</td>
</tr>
<tr>
<td></td>
<td>Control*</td>
<td>15.80 (12.50–19.10)</td>
<td>ND</td>
<td>9.82 (5.92–13.72)</td>
</tr>
</tbody>
</table>

ND = Not determined. *Mean (range) for 2 control cats.

Discussion

To aid in the development of a vaccine for animals, heifers in 1 study were immunized against LH-releasing hormone, hCG, and bovine LH. Additionally, male cats and dogs have been immunized against LH-releasing hormone conjugated to tetanus toxoid, and male mice have been immunized with LH-releasing hormone fusion proteins. Immunization in males results in a decrease in serum testosterone concentrations to values similar to those achieved after castration, which results in azoospermia. Investigators observed that active immunization with LH-R suppressed fertility of male rats and increased circulating maternal antibodies against LH-receptors. Female rats passively immunized with LH-R antibodies produced in rabbits displayed constant estrus and failed to conceive after repeated matings. Those female rats resumed normal estrous cycles and had normal fertility 1 month after the last injection of LH-R antibodies. Pregnant mice injected with LH-R monoclonal antibodies developed in monkeys produced only 3 viable pregnancies and 10 offspring, compared to 8 pregnancies and 45 offspring born to normal control mice. In another study, female rabbits actively immunized with bovine LH-R failed to ovulate and did not conceive after repeated mating and artificial insemination. Similarly, baboons and rhesus monkeys actively immunized with bovine LH-R remained infertile for up to 1 year, but they returned to normal fertility after antibody titers decreased.

Composites of mean hormone concentrations in immunized and control cats revealed that serum E2 concentrations in immunized cats did not differ significantly from concentrations for the control cats, indicating basal follicular growth and a lack of estrogen deprivation in the immunized cats. It may be speculated that E2 may have a dominant role in the regulation of pituitary LH secretion and P4 negative feedback in cats. There was little difference in the serum LH concentrations of immunized and control cats. However, a surge of LH could not be detected, because blood samples were not collected daily. A surge in serum LH concentrations in response to administration of LH-releasing hormone suggests that the hypothalamic-pituitary axis remained intact in the immunized cats.

Serum P4 concentrations were suppressed in the immunized cats with LH-R antibodies. These observations indicated that circulating LH-R antibodies directly inhibited binding of LH to ovarian receptors and, thus, production of P4 despite typical reserves of LH in the pituitary gland. Immunized cats did not display signs of estrus. On the other hand, episodic increases in P4 concentrations were evident on day 139 in 1 control cat and days 83 and 180 in the other control cat (Fig 2), indicating that the cats had presumably ovulated during the study.

We do not believe that immunized cats were induced to ovulate by administration of LH-releasing hormone on day 345 after implantation. Suppression of P4 in the immunized cats apparently because of circulating LH-R antibodies indicated a lack of ovulation or poor corpus luteum function. With the decline in the LH-R antibody titers (approx day 501), immunized cats began to have evidence of restoration of typical physiologic ovarian status, suggesting that the immunized cats did not mount an autoimmune response. However, these cats were not mated to assess fertility, and histologic examinations were not performed to assess the functional state of reproductive organs in this study. The control cats had an increase in serum P4 concentrations followed by an increase in estrogenic influences accompanied with estrus and vaginal cells consistent with estrus, indicating fertile status of cats during the entire study period. Initially, 3 cats were included in the control group. However, 1 cat died during a preliminary stage of the study. The remaining 2 control cats were monitored throughout the entire study period of 516 days.

Active immunization against LH-R in baboons and female dogs resulted in serum E2 concentrations that were within the respective reference range, and a reciprocal relationship between LH-R antibody concentrations and serum P4 concentrations was observed. Twelve months after LH-R implantation, animals in those studies returned to a normal physiologic pattern consistent with fertility.

The antibody response in the study reported here revealed considerable variation among immunized cats. Because of the limitations of the experimental design, booster injections were administered arbitrarily at the time a decrease in antibody titers was anticipated in an effort to sustain the immune response. The study did not include a dose-response design; therefore, it was not possible to determine an optimum titer of LH-R antibody that would cause infertility. Histologic examination of reproductive organs and results of mating, including determination of conception rate and litter size, will have to be performed to establish optimum dose-response relationships between antibody titers and ovarian function. However, analysis of the results of the study reported here revealed that LH-R antibody produced in cats in response to immunization against bovine LH-R, even at low antibody titers, could suppress P4 concentrations. The effect of immunization against LH-R on P4 secretion indicates a lack of an LH effect on the dominant follicles attributable to antibodies against LH-R resulting in anovulation or poor corpus luteum function.
Results of the study reported here and other studies apply document the potential for a recombinant LH-R vaccine as an immunomodulator of gonadal function, which may have application in regulation of fertility in females and males. The clinical implications of an immunoncontraceptive vaccine based on inoculation with LH-R can be recognized in the observation that the effect is reversible, as opposed to the final and irreversible effects of ovariohysterectomy or neutering. Also, the negative effect on development of mammary gland tumors seen in female dogs after long-term application of synthetic progestogens for the prevention of estrus and pregnancy can be avoided. Because of extensive structural homology of LH-R among species and cross-species nonspecificity of antibodies against LH-R, an antifertility vaccine based on inoculation of LH-R may have application in cats and other vertebrates.

References