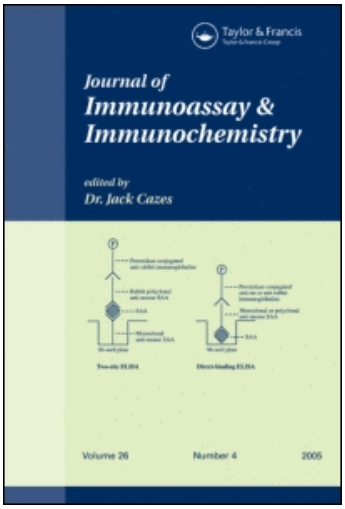

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Effect of Immunization with Lutropin-Receptor on the Ovarian Function of Rabbits

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EFFECT OF IMMUNIZATION WITH LUTROPIN-RECEPTOR ON THE OVARIAN FUNCTION OF RABBITS[1]

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Abstract

Three New Zealand white adult female rabbits, designated as A, B, and C, were immunized to produce lutropin receptor antibodies. Antisera inhibited the binding of ^{125}I-hCG to the lutropin receptor and the production of testosterone by hCG stimulated rat Leydig cells. During the study period of, approximately 10 months after the last immunization, rabbits did not ovulate in response to an injection of 75 IU of human chorionic gonadotropin or mating as revealed by laparotomy. As the antibody titers declined, induction of ovulation and laparotomy revealed restoration of ovulation and corpus luteum formation. However, no pregnancy occurred when rabbits A and B were mated and artificially inseminated. These observations indicate that lutropin receptor antibodies can cause infertility in female rabbits.

Key Words: Lutropin receptor: Antibodies: Ovarian function.

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Introduction

Specific receptors, common to both lutropin (LH) and human chorionic gonadotropin (hCG), are present in the testicular and the ovarian cells and mediate the action of LH on the gonads as shown by Dufau et al. (1), Wardlaw et al. (2), Lee et al. (3), Pandian and Bahl (4), Dattatreyamurty et al. (5), Metsikko and Rajaniemi (6), and Haour and Saxena (7). Antibodies against LH-receptor have been produced in rabbits (5-7). The purpose of this study was to immunize rabbits against bovine LH receptor to produce antibodies and to observe the effects of circulating antibodies on the ovarian function and fertility of female rabbits.

Materials and Methods

Immunization :

Bovine LH-receptor (LH-R) with a binding capacity of approximately 3,000 pM/mg protein (5,8) was used to immunize three New Zealand white adult female rabbits designated as A, B and C. Prior to immunization, one mL aliquots of blood were collected from the ear vein of each rabbit as the control. Untreated rabbits in the colony were also used as controls. An aliquot of 100 ug protein equivalent of receptor was suspended in 100 uL of normal saline, emulsified in 100 uL of complete Freund's adjuvant and used for the initial immunization by intradermal injections of 10-20 uL each at multiple sites. Subsequently, aliquots of 100 ug of receptor emulsified in the adjuvant were injected subcutaneously at four week intervals for a period of five and a half months. Rabbits were bled by ear

vein puncture at four week intervals. The blood was centrifuged at 3,400 rpm for 15 min. at 4°C and serum stored at -20°C until analyzed for lutropin receptor antibody titers, estradiol (E_2), progesterone (P) and lutropin (LH) levels. The gamma-globulin fraction of the sera was isolated by the Rivanol precipitation method of Horejsi and Smetana (9) and concentrated by ultrafiltration through an Amicon PM-10 membrane. The gamma-globulin fraction was gel-filtered through a 3 x 30 cm column of Sephadex G-25 (fine), equilibrated and eluted with 0.1 M ammonium bicarbonate buffer of pH 8.5. The gamma-globulin, eluted in the unretarded fraction, was lyophilized and stored at 4°C. The protein concentration in the gamma-globulin fraction was determined by the method of Lowry et al., (10). Rabbit sera and the gamma-globulin fractions were tested for the presence of antibody by the microplate enzymeimmunoassay (ELISA) as described by Munro and Stabenfeldt (11), by the inhibition of ^{125}I-hCG binding to the receptor, and by the inhibition of testosterone production by hCG stimulated rat Leydig cells (14).

Antisera were also examined for binding with ^{125}I-FSH, ^{125}I-hCG, and ^{125}I-hLH in a competitive protein binding assay. Antisera were absorbed with the receptor or hCG as follows (Table I). One mL aliquots each of antisera were mixed, separately in duplicate, with 0.5 ug, 2.5 ug, 5 ug and 10 ug of receptor as well as with 6.25 ug, 12.5 ug, 50 ug, and 100 ug of hCG containing 12,000 IU/mg and incubated for 3 hrs. at 37°C. The incubates were centrifuged at 3,400 rpm in a Sorvall refrigerated centrifuge for 20 min. The supernatants were collected separately and analyzed for antibodies to the receptor and hCG. The immunoreactivities of the native unadsorbed and absorbed LH-R antibodies receptor, of its dimer, of its monomer, and of its

TABLE I

Percent binding of unabsorbed LH-R antisera[1], LH antisera absorbed with LH-R, and LH-antisera absorbed with hCG to ^{125}I-LH-R and ^{125}-I-LH/hCG

	^{125}I-LH-R % bound		^{125}I-LH/hCG % bound
LH-R Antiserum (unabsorbed)	55.7		55.7
Absorbed with LH-R[2]	% bound	Absorbed with hCG[3]	% bound
0.5 ug	15.3	6.25 ug[4]	44.8
2.5 ug	10.3	12.5 ug	34.3
5.0 ug	4.4	25.0 ug	20.2
10.0 ug	3.0	50.0 ug	9.3

[1] LH-R dilution 1:2000; ^{125}I-FSH did not bind to LH-R antibody.

[2] hCG did not compete with the ^{125}-LH-R for binding to the receptor.

[3] LH-R did not compete with ^{125}I-hCG for binding to the receptor.

[4] hCG contained 12,000 IU/mg.

subunits were determined quantitatively in the radioimmunoassay system as described below.

Inhibition of ^{125}I-hCG Binding to the Receptor:

Approximately 500 ug of lyophilized bovine corpora lutea plasma membranes (5) were suspended in 100 uL of distilled water and incubated with approximately 50,000 cpm (approximately 1 ng) of the ^{125}I-hCG in 100 uL of 10 mM Tris-HCl buffer of pH 7.2 containing 0.1% BSA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.01% NaN_3, at 4°C for 1 hr. in the presence of 100 uL of various dilutions of antisera and of gamma-globulin fractions in 100 uL of 0.05 M

phosphate buffered saline (PBS) of pH 7.4, containing 0.1% BSA, 0.01% NaN_3 and 0.2% EDTA. Controls containing normal rabbit serum and normal rabbit gamma-globulin were analyzed simultaneously.

Radioimmunoassay of LH-Receptor:

Highly purified LH-hCG receptor (12) was iodinated by the Chloramine-T method of Hunter & Greenwood, 1962 (13) with minor modifications as described below. An aliquot of 50 uL of 0.5 M sodium phosphate buffer of pH 7.4 was added to 5 ug of highly purified LH-hCG receptor in 5 uL of 0.1 M sodium phosphate buffer of pH 7.4 containing 0.1% Triton X-100. To this were added 1 mCi of $Na^{125}I$ and 20 uL of a solution of 1 mg of Chloramine-T/mL solution in phosphate buffer. The reaction was stopped after 5 seconds by the addition of 50 uL of sodium metabisulfite (2 mg/mL solution in the phosphate buffer) followed by the addition of 0.5 mL of 0.1 M sodium phosphate buffer of pH 7.4 containing 0.4% Triton X-100. The labeled receptor was separated from free ^{125}I by gel filtration on a 1 x 50 cm column of Ultrogel AcA-34, eluted by 0.1 M sodium phosphate buffer containing 0.1% Triton X-100. A nonequilibrium radioimmunoassay with a double antibody method of separation of bound and free ^{125}I-receptors was used as described below.

The "RIA buffer" consisted of 125 mM phosphate buffer of pH 7.0 containing 0.02 M EDTA, 0.1% Triton X-100, and 0.1% human serum albumin. The total count (TC) tube contained only 100 uL of the ^{125}I-receptor solution, and the blank tube contained 100 uL of the ^{125}I-receptor and 150 uL of the RIA buffer. The antiserum to the receptor (50 uL, initial dilution of 1:2000) was incubated either with 50 uL of the RIA buffer for total binding

(TB) or with known amounts of 5 mg/mL solution of the LH-hCG receptor for total displacement (TD) for 24 h at 4°C. At the end of the incubation, 100 uL of the ^{125}I-LH-hCG receptor solution (100,000 cpm, specific activity 43-50 uCi/ug) was added to the tubes. The tubes were incubated further for 24 h at 4°C. The antibody, viz., goat anti-rabbit-gamma-globulin (50 uL), was added to all TB and TD tubes and mixed. The tubes were centrifuged for 30 min at 3500 rpm. The supernatants were aspirated and the radioactivity was counted in an Autogamma counter (Micromedic with 62% efficacy).

Inhibition of Testosterone Production by Rat Leydig Cells:

Sprague-Dawley male rats between the age of 56-70 days and of body weight range of 250-300 g were used to obtain Leydig cells. Testes were dissected out, teased, gently homogenized, and suspended in medium 199 with 26 mM Hepes buffer, Hank's salts, L-glutamine (GIBCO) containing 0.125 mM of 1-methyl-3-isobutylaxanthine (Sigma), and 0.1% BSA. The homogenate was gently stirred on a magnetic stirrer on ice for 15 min then filtered through a nylon mesh into a siliconized glass conical flask and preincubated for 30 min. at 34°C under 95% O_2 and 5% CO_2 in a metabolic shaker at 150 cycles/min. The Leydig cell suspension was centrifuged at 120 x g. Supernatant was discarded and the pellet containing Leydig cells was resuspended in fresh 10 mL of the same media containing 2% calf serum (GIBCO) at a concentration of 10 ml per pair of testes and used as the source of receptor. The assay was performed as described by Dufau et al., (14) with minor modifications. Aliquots of 200 uL of the Leydig cell suspension were added to siliconized assay tubes and preincubated with 50 and 100 ug of normal rabbit-gamma-globulin

obtained from serum prior to immunization, as control, as well as with 50 and 100 ug immunoglobulin obtained from antiserum against LH receptor in duplicate at 34°C under 5% CO_2 and 95% O_2. One hundred uL of a solution of 2 mIU/mL (10,000 IU hCG/mg) was then added to the tubes of each dilution and further incubated for 2 h. The incubates were centrifuged and testosterone levels were measured in the supernatants by a radioimmunoassay (RIA) kit obtained from Diagnostic Products Corporation, Los Angeles, CA. The results of the rat Leydig cell assay were expressed as percent inhibition of testosterone production of the control.

Determination of E_2, P and LH Levels in Rabbit Serum Samples:

The serum samples obtained from rabbits prior to and after the last immunization were analyzed for E_2 and P levels by a radioimmunoassay kit obtained from Nuclear Medical Systems, Inc., Newport Beach, CA. Lutropin levels in rabbit sera were estimated by RIA. Rabbit LH was supplied by National Hormone and Pituitary Program, Baltimore, MD. Rabbit LH was iodinated by the Chloramine T method (13) and was also used as the standard.

Effect of Immunization on Ovarian Function:

The effects of active immunization against the LH receptor on the reproductive function of the rabbits were observed by the determination of LH-receptor antibody titers in the serum samples drawn at various intervals by the inhibition of ^{125}I-hCG binding to the LH-R. The rabbits were induced to ovulate and then artificially inseminated and/or mated. Laparotomy was performed to observe follicular growth, ovulation, corpus luteum formation as well as implantation. As shown in Fig. 1 for rabbit A, two months after the last immunization, 75 IU of hCG was injected via

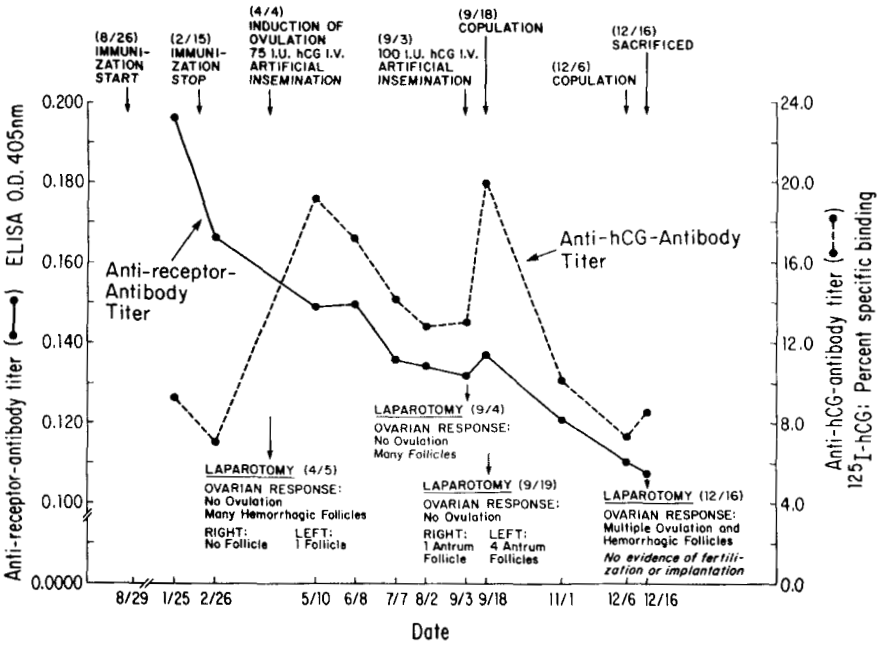

Fig. 1. Effect of immunization with lutropin receptor on the ovarian function in rabbit A.

ear vein to induce ovulation. Rabbit A was immediately artificially inseminated with 2 mL of fresh semen obtained from the epididymis of a fertile male rabbit. The laparotomy was performed 20 hrs later. The ovaries were examined for the presence of follicles and ovulation. The uterii were examined for implantation sites. Five months later, rabbit A was again induced to ovulate by the injection of 100 IU of hCG, inseminated and another laparotomy was performed. Two weeks later, the rabbit A was mated with two fertile males, and followed by laparotomy. Finally, two weeks later the rabbit A was again mated and laparotomy was performed.

Similarly, as shown in Fig. 2, for rabbit B, seven months after the last immunization rabbit B was mated and laparotomy was

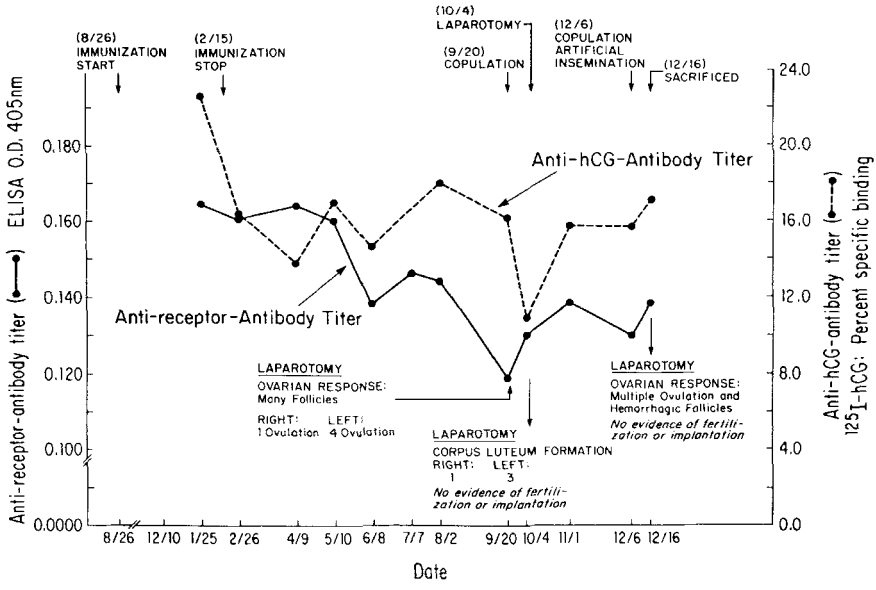

Fig. 2. Effect of immunization with lutropin receptor on the ovarian function in rabbit B.

performed 24 hours later to observe the status of the ovaries and uterii. Another laparotomy was performed two weeks later. Two months later, rabbit B was again mated as well as artificially inseminated and laparotomy was performed two weeks later. Rabbit C was treated similar to rabbits A and B. Rabbit C was sacrificed. Blood was collected via cardiac puncture. Tissues from brain, lung, heart, thyroid, spleen, stomach, kidney, liver, intestine, uterus, and ovary were removed and fixed in 40% formalin for histopathological examination.

Results

Circulating antibodies to LH-receptor were detected in all three rabbits within 8-12 weeks and the antibody titers in the

sera of all three rabbits increased from 1:100 to 1:2,000 dilution to cause approximately 50% inhibition of the binding of ^{125}I-hCG to the receptor. Sera collected prior to immunization did not inhibit the binding of ^{125}I-hCG to the receptor. One hundred ug of gamma-globulin, caused 30±10% inhibition of the binding of ^{125}I-hCG to bovine corpora lutea membranes and 150 ug and 300 ug of gamma-globulin caused, respectively, 67±5% and 85±5% inhibition of binding of ^{125}I-hCG to rat Leydig cells in three separate assays. One hundred ug of gamma-globulin at 50 ug and 100 ug dose levels of immunoglobulin also caused an average of 28±6% and 40±10% inhibition in the production of testosterone by hCG-stimulated rat Leydig cells.

The LH-receptor antibodies did not bind to ^{125}I-FSH. They however did bind ^{125}I-hCG and ^{125}I-LH. The antiserum absorbed with 0.5 ug, 2.5 ug, 5 ug, and 10 ug of receptor showed a progressive decrease in the binding of the ^{125}I-receptor from an average of 55.7% with unabsorbed serum to 15.3%, 10.3%, 4.4% and 3%, respectively, with the absorbed serum. However, binding of ^{125}I-hCG to the unabsorbed and absorbed antisera receptor did not change. The ^{125}I-receptor bound to the absorbed antiserum was not displaced by unlabeled hCG. Similarly, after the absorption of the LH-receptor antisera with 6.25 ug, 25 ug, 50 ug and 100 ug of hCG per mL, the specific binding of ^{125}I-hCG to absorbed sera decreased from an average of 53.8% of the unabsorbed serum to 44.8%, 34.3% and 20.2%, respectively, of the absorbed serum (Table I). The ^{125}I-hCG bound to the absorbed antiserum was not displaced by unlabeled receptor.

Serum Levels of E_2, P and LH:

Hormonal analyses were performed on the pool of rabbits' sera, prior to initial immunization and after the last

immunization. Average and SEM of three determinations of serum estradiol level after immunization was 71±4.25 pg/ml as compared to an average of 75±5.6 pg/ml prior to immunization. The average LH levels in serum after the last immunization, being <2.5 ng/ml, were significantly lower than 9.0±3.2 ng/ml, prior to immunization. Average serum progesterone level of 0.45 ng/ml±0.14 in immunized rabbits was also significantly lower than the average of 4.7±0.9 ng/ml prior to immunization.

Effect on the ovarian function:

As shown in Fig. 1 for rabbit A, two months after the last immunization, induction of ovulation by hCG injection followed by artificial insemination showed no evidence of ovulation at the time of laparotomy. However, the left ovary contained one follicle. Uterii were normal in size and appearance. Seven months after the last immunization, the receptor antibody titer declined to significantly lower levels. At that time a repeat induction of ovulation and artificial insemination followed by another laparotomy revealed the ovaries to be of normal size with many follicles, with no evidence of ovulation. Two weeks later, the rabbit was mated and another laparotomy was performed after 24 hrs. The right ovary had one and the left ovary had four antrum follicles. There were no signs of ovulation. Two and a half months later, the antibody titers declined further. The rabbit was mated again and a laparotomy was performed the next day which revealed multiple ovulation and hemorrhagic follicles, however there was no evidence of fertilization or implantation.

As shown in Fig. 2, rabbit B was mated with fertile males, approximately seven months after the last immunization followed by laparotomy which revealed normal ovaries with many normally

growing follicles. There was evidence of one ovulation in the right and four in the left ovary. Repeat laparotomy two weeks later showed one corpus luteum in the right and three corpora lutea in the left ovary, however, no evidence of fertilization or implantation was found. Two months later, Rabbit B was again mated as well as artificially inseminated. Two weeks later, laparotomy revealed multiple ovulation and hemorrhagic follicles with no evidence of fertilization or implantation. As the LH-receptor antibody titer declined to lower levels, there was a concomitant reversal of the ovarian function towards normal follicular growth, ovulation and formation of corpora lutea. During, the period of approximately 10 months neither mating nor artificial insemination, resulted in pregnancy. Rabbit C showed responses similar to rabbit A and B and was sacrificed for histopathological examination of tissues from various organs which showed no apparent adverse effects.

Discussion

The antigenic nature of the LH-receptors is well established. Autoantibodies against LH-receptor have been implicated by Saxena and Singh as one of the possible causes of premature ovarian failure (15). In previous studies by Saxena and Singh (16), and Pal et al., (17) active immunization of baboons with LH-receptor caused infertility due to the production of antibodies. However, the animals returned to normal ovarian function with the decline in the antibody levels.

In this study, rabbits were immunized against bovine LH-receptor to generate antibodies. In addition to LH-receptor antibody, antibodies to ^{125}I-hCG and ^{125}I-LH were formed. No

antibodies to FSH were generated. There was a lack of displacement of bound ^{125}I-receptor by hCG as well as by the lack of displacement of bound ^{125}I-hCG by the receptor. Absorption of LH-receptor antiserum with the receptor or hCG removed the irrespective antibodies in a dose response manner. The serum absorption studies suggested that antibodies reactive to hCG were elicited by immunization with lutropin receptor molecules. This finding implies that anti-idiotypic antibodies (AB-2) were generated against receptor epitopes that immunologically resembled an epitope or epitopes on hCG. The hypervariable region of these immunoglobulin molecules might have then represented an antigen to which the rabbits could have elicited an antibody response to produce an antibody (AB-3). Such antibodies (AB-3) would appear as anti-hCG antibodies and may explain the binding of lutropin receptor antibodies with ^{125}I-hCG and ^{125}I-LH. These data indicates that three separate populations of antibodies were generated due to chronic immunization of the rabbits with LH receptor, namely AB-1 against LH receptor, idiotypic AB-2 against lutropin receptor antibody and the antidiotypic AB-3 which reacted with ^{125}I-hCG and ^{125}I-LH. This is further supported from the observation that injections of hCG in rabbit A (Fig. 1) appeared to boost anti-hCG titers.

As shown in Fig. 1 and 2, after the last immunization, the injection of hCG in rabbit A and artificial insemination and/or mating in rabbit B did not induce ovulation but follicular growth occurred as revealed by laparotomy. With the decline in the antibody titers, injection of hCG and mating induced multiple ovulation and showed hemorrhagic follicles with no evidence of ovulation, fertilization or implantation and the uterii appeared normal. Rabbit C also showed similar ovarian response. The

rabbit did not show any toxic effects or tissue histopathology due to the presence of LH-receptor antibody in the circulation.

It may be noted that the estradiol levels in the immunized rabbits remained in the normal range which is consistent with the folliculogenesis, whereas the serum levels of LH decreased, perhaps due to a partial neutralization of endogenous rabbit LH by idiotypic antibodies (AB-3) causing poor corpus luteum function and consequently low levels of progesterone.

These observations demonstrate that circulating anti LH receptor antibodies caused ovarian dysfunction and infertility in female rabbits without apparent adverse effects. However, as the antibody titers declined, normal ovarian function was restored. Presence of antibodies binding with ^{125}I-hCG and ^{125}I-LH may suggest a role of antibodies against LH and/or hCG in causing infertility. However, it has been known for a long time that antibodies against hCG do not reduce fertility in rabbits as described by Glass and Mrouch (18). It is, therefore, likely that ovarian dysfunction and infertility was due to circulating antibodies against the LH-receptor (19), which was further born out by the occurance of pregnancy in rabbits A and B, when the antibody titer were undetectable. Although further characterization of the LH-receptor antibodies and its effects on the ovarian function in a more controlled study design is warranted, the present observations suggest that antibodies against LH-receptor may cause immunological infertility in rabbits and could be useful agents in fertility regulation of domestic animals.

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