GnRH or eCG treatment fails to restore reproductive function in GnRH immunized ewes

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Received 22 February 2008; received in revised form 2 April 2008; accepted 23 April 2008
Available online 2 May 2008

Abstract

This study was designed to evaluate the potential of using eCG or GnRH in restoring reproductive functions in GnRH immunized ewes. Thirty-three multiparous Kivrıcık ewes were randomly assigned into either control group (n = 11) or immunization group (n = 22). Ewes were immunized against GnRH by injecting with a cocktail of ovalbumin-LHRH-7 (ovalbumin-GnRH-7) and thioredoxin-LHRH-7 (thioredoxin-GnRH-7) fusion proteins generated by recombinant DNA technology in April. 500 IU eCG or 0.008 mg GnRH analogue was used to induce ovulations. Serum GnRH antibodies were present in animals of the immunized group beginning the second week after the first immunization and maintained throughout the study (14 months). Immunization caused anestrus in immunized ewes. eCG or GnRH analogue administration given after 14 days progestagen (20 mg fluorogestone acetate, FGA) treatment during breeding season (mid July) did not induce ovulation in these ewes. Two more attempts with single or multiple eCG injections failed to induce ovulation in this group as well. It appears that the gonadotropin stimulation was not of adequate time since neither eCG nor GnRH administration was able to restore reproductive function in immunized animals. The immunization effect lasted more than a year. These results suggest that GnRH immunization exerts its effect via the hypothalamo-pituitary axis and that more than such stimulation is required to overcome the reproductive suppression.

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Keywords: GnRH; Fusion proteins; Immunization; Ewes

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1. Introduction

Immunization against gonadotropin releasing hormone (GnRH) has been described as one of the methods to reduce reproductive functions in farm animals and a possible alternative to surgical castration (Reeves et al., 1989; Bonneau and Enright, 1995; Thompson, 2000). Gonadotropin hormone concentrations, testicular development and sexual activities are suppressed in GnRH immunized animals (Robertson et al., 1982; Hoskinson et al., 1990; Adams and Adams, 1992). Two recombinant fusion proteins, ovalbumin-LHRH-7 (ovalbumin-GnRH-7) (OL) and thioredoxin-LHRH-7 (thioredoxin-GnRH-7) (TL), were developed to be used as a sterilization vaccine (Zhang et al., 1999; Quesnell et al., 2000). The effectiveness of these recombinant proteins in suppressing reproductive functions was demonstrated in heifers (Sosa et al., 2000), bulls (Aissat et al., 2002) and ram lambs (Ulker et al., 2001; Ulker et al., 2005). The effectiveness of these recombinant proteins in suppressing reproductive functions in ewes has not been studied.

Many researchers report that active immunization against GnRH induces only a temporary suppression of reproductive functions after which animals return to normal fertility (Reeves et al., 1989; D’Occhio, 1993; Bonneau and Enright, 1995; Thompson, 2000). Alternatively, reproductive functions in GnRH immunized animals could be restored by using GnRH agonist that did not cross-react with the antibodies (Adams and Adams, 1986; Herman and Adams, 1990; Sakurai et al., 1992), multiple injections of FSH, LH, hCG (Mariana et al., 1998) or eCG (Oatley et al., 2005). Nevertheless, single injection of GnRH to restore reproductive functions after active immunization done in early life (Brown et al., 1994, 1995; Clarke et al., 1998) or adult age in ewes (Jeffcoate et al., 1978) failed to do so. Immunizing farm animals against GnRH allows animal owners to keep the male and female animals together as long as the immunization effect lasts. Restoring reproductive functions in immunized animals without waiting for the decline in circulating anti-GnRH antibodies below a threshold required to neutralize GnRH could be a useful management tool. Nevertheless, no such studies related to restoring reproductive function after active immunization against GnRH at adult age have been shown. In sheep immunized against GnRH at prepubertal or peripubertal age, plasma luteinizing hormone (LH) concentrations were not restored after GnRH injection at a time when anti-GnRH antibodies are low (Brown et al., 1994, 1995) or not detectable (Clarke et al., 1998). These findings have led to the suggestion that in the young ewe the basal hypothalamus-medial eminence is a target site for anti-GnRH antibodies which cause lesions disrupting the integrity of hypothalamus and, consequently, long term reproductive suppression. Findings in swine support this suggestion (Molenaar et al., 1993). D’Occhio et al. (2001) suggest the presence of a long-term permanent effect in bulls immunized at adult age. Conversely, Mariana et al. (1998) were able to induce preovulatory follicles in ewes immunized against GnRH early in life by multiple injections of FSH, LH or hCG. Similarly, spermatogenesis has been restored in GnRH immunized bulls using eCG administered IM every 2 week for 80 days (Oatley et al., 2005). It is not known whether reproductive functions could be restored by eCG injection in ewes actively immunized against GnRH at an adult age using recombinant fusion proteins.

Suppression of LH, which is a result of GnRH immunization, has been suggested to cause early disruption of pregnancy through inadequate pituitary support to corpus luteum (CL) (Tast et al., 2000). So, even though reproductive functions are restored in immunized animals using gonadotropin hormones, i.e. eCG, there is a possibility that pregnancy might be ended because of circulating anti-GnRH antibodies neutralizing GnRH and consequently low levels of LH. However, considering the longer half life and LH like effect (Murphy and Martinuk, 1991), eCG...
could provide adequate LH support during the early days of pregnancy. If ovarian functions of GnRH immunized animals can be induced by eCG administration, then, valuable information could be obtained on establishment and maintenance of pregnancy under high anti-LHRH antibody concentrations.

The purposes of this study were (1) to determine the effectiveness of recombinant LHRH fusion proteins in suppressing reproductive functions in mature, pluriparous ewes and (2) to investigate the possibilities of using eCG or LHRH in restoring reproductive functions in LHRH immunized ewes.

2. Materials and methods

2.1. Animals and treatments

Thirty-three multiparous Kivircik ewes, a multi-purpose (meat, milk and wool) thin-tailed local sheep breed of Turkey, at 3–8 years of age and weighing average 39 kg were randomly assigned into control \( (n=11) \) and immunization \( (n=22) \) groups. All ewes were subjected to estrous synchronization program consisting of 14 days progestagen (20 mg FGA) impregnated sponge plus 500 IU eCG injection at sponge withdrawal at least once previously. Breeding season starts in June and majority of the ewes are mated during July in Cine district (latitude 37°61’E, longitude 28°06’N) of Aydin region, Turkey. Therefore, the immunization schedule was designed to obtain high antibody concentrations during the mating period. Thus, ewes in the immunization group were immunized against GnRH using GnRH fusion proteins on April 11, which was 111 days prior to proposed mating time. Since all ewes were being suckled during that time in order to eliminate the possibility that suckling may suppress ovarian activity, ewes were subjected to a ‘21-day decreasing suckling program’ beginning 1 week after immunization to get them dry. Booster immunization was done 1 month after the first immunization.

2.2. Preparation of antigens and immunizations

OL and TL proteins were produced from previously constructed ovalbumin-GnRH-7 and thioredoxin-GnRH-7 genes generated by recombinant DNA techniques as described by Zhang et al. (1999) and Quesnell et al. (2000), respectively. Recombinant genes were over expressed in Escherichia Coli. His-bind affinity chromatography using a Ni\(^{2+}\) column allowed for purification of the proteins. Equimolar amounts of each GnRH fusion protein (10 nM) totalling 0.75 mg of protein were suspended in 6 M urea and emulsified in 0.5 ml of modified complete Freund’s adjuvant (Sigma, St. Louis, MO, USA) for the primary immunization and incomplete Freund’s adjuvant was used for the booster injection. Immunizations were distributed over four subcutaneous sites on the inside surface of the legs.

2.3. Data collection

Two weeks after the booster a harnessed teaser ram was introduced to stimulate sexual functions. Behavioral estrus was observed. Ninety-five days after the first immunization all animals were subjected to a typical estrus synchronization program: Progestagen (20 mg fluorogestone acetate, FGA) impregnated sponges (Chronogest®©, Intervet International, Boxmeer, The Netherlands) were inserted for 14 days. At sponge withdrawal control animals and 11 animals in immunization group were administered with 500 IU eCG, with i.m. injection. The other 11 ani-
mals in immunization group were administered i.m. with 0.9 ml/animal GnRH analogue (0.008 mg Buserilin [Receptal]; Hoechst UK Ltd., Milton Keynes, UK). Two days after eCG or GnRH analogue injections rams were introduced. One ram was assigned per 4–5 ewes in separate pens. Heat and mating activities were observed (Fig. 1).

Since no estrus activity was observed in the immunized ewes 10 days after sponge removal all animals in this group were injected i.m. with 400 IU eCG to induce ovulation (second attempt). Rams introduced after sponge withdrawal remained with ewes until pregnancy determination (Fig. 2).

All animals we examined for pregnancy using 6 MHz linear rectal probe (Pie medical, 100 Falco vet.) 35 days after sponge withdrawal. Since no animals were pregnant in immunization group a ‘long term ovarian stimulating program, multiple eCG injections’ was initiated (third attempt). For this purpose 14 animals in immunization group were injected i.m. with 400 IU eCG once and then 280 IU eCG i.m. three times at 6 days intervals and once 3 days after forth one.
Progestagen containing sponges were inserted at the third eCG injection and removed at the last injection. At sponge removal fertile rams were introduced and estrus behaviors were observed. The rams remained with ewes all time (Fig. 2). All control and 14 animals from immunized group were kept with fertile rams during the subsequent year’s mating season.

Beginning from the first immunization all animals were bled every 3–4 days to monitor levels of ovarian hormones. Also all animals were bled every 2 weeks to determine anti-GnRH antibody concentrations. For the last 7 months of the study, only monthly blood collection was performed. Blood samples were refrigerated overnight at +4 °C, and then centrifuged at 3000 rpm for 15 min. Serum was harvested and stored at −20 °C until subsequent analyses.

A radioactive binding assay was used to evaluate the percentage of $^{125}$I-GnRH that would bind to the anti-GnRH antibodies present in the serum at a 1:1000 dilution. Mouse anti-sheep gamma globulin was used as the second antibody at 1:20 dilution. Iodination of GnRH was performed using GnRH (2.5 μg/25 μl H2O) with 0.5 mCi (5 μl) $^{125}$I, 30 μl of 0.5 M PBS (pH 7.5) and 10 μl Chloramine-T (600 ng/10 μl of 0.5 M PBS, pH 7.5). I-GnRH was separated on a QAE-Sephadex column using column buffer (10 mM Tris, 1 mM CaCl2; 0.1% BSA, pH 7.2). The percentage of $^{125}$I-GnRH bound to serum diluted 1:1000 was used as the index for GnRH antibody titers.

Serum progesterone concentrations were determined by RIA (DSL-3400, Diagnostic Systems Laboratories, Inc. Webster, TX). All procedures were conducted according to the manufacturer’s instructions.

Data analysis was performed using GLM procedure of SAS (SAS Inst. Inc., Cary, NC) for repeated measures to determine main effects of treatment, time and treatment × time for each of response variables (serum anti-GnRH antibody percentage bound, progesterone concentration). Data are presented as means ± S.E.M.

### 3. Results

GnRH antibodies were detected within 2 weeks after the first immunization. The booster immunization caused an increase in antibody production. GnRH antibodies formed a plateau beginning 2 months after the booster immunization and remained at the same level for 11 months until the end of the study (Fig. 3).

Cyclicity of the ewes was determined from progesterone concentrations. Ewes having higher progesterone concentrations for at least three consecutive samplings taken at 3-day intervals were considered as cycling. Accordingly, progesterone profiles showed that it is most likely all ewes were cycling.

![Fig. 3. Mean (±S.E.M.) antibody binding to GnRH expressed as a percentage bound $^{125}$I-GnRH at 1:1000 dilution in control and immunized ewes (S.E.M. ± 5.4 and 3.1, respectively). Arrows represent age of immunizations.](image-url)
Reproductive traits in control and immunized ewes after a traditional estrus synchronization program (first attempt to restore reproduction)

<table>
<thead>
<tr>
<th></th>
<th>Ewes exhibited estrus behavior</th>
<th>Pregnant ewes at 35 days</th>
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<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Immunization + eCG</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Immunization + GnRH</td>
<td>0</td>
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were in anestrus when the first immunization was given. Teaser ram introduction in late May did not induce ovulations and estrous behavior in either group. Gradually animals in control group started cycling as the breeding season progressed. Before the synchronization program was initiated all ewes in control group were cycling, while all ewes in immunization group still were in anestrus (Fig. 4).

All ewes in control group showed behavioral estrus 1–2 days after sponge removal and were mated with fertile rams (first attempt). None of the ewes in immunization group exhibited behavioral estrus after sponge withdrawal. All ewes in control group were diagnosed as pregnant at 35 days after sponge withdrawal and lambed at term whereas none of the ewes in immunization group were diagnosed at this time (Table 1).

Immunized animals did not exhibit estrus after eCG injection given 10 days after the first eCG or GnRH injections (second attempt). No estrous behavior was observed in any of these ewes, except one individual, at the end of multiple eCG injections accompanied with FGA containing sponge (third attempt) (Table 2). None of the ewes in immunization group were pregnant even though they were kept with fertile rams more than 2 months in which breeding season ended.

Reproductive traits in immunized ewes subjected to single or multiple eCG injections (second and third attempts to restore reproduction)

<table>
<thead>
<tr>
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<th>Single eCG administration (11 animals)</th>
<th>Multiple eCG administration + FGA (14 animals)</th>
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<tbody>
<tr>
<td>Ewes exhibited estrus behavior</td>
<td>Pregnant ewes</td>
<td>Ewes exhibited estrus behavior</td>
</tr>
<tr>
<td>Immunization</td>
<td>0</td>
<td>1</td>
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</tbody>
</table>
Table 3
Pregnancy results in control and immunized ewes for 2 years.

<table>
<thead>
<tr>
<th></th>
<th>First year</th>
<th>Second year</th>
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<tr>
<td></td>
<td>Lambed ewes</td>
<td>Lamb numbers per ewe</td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>1.4</td>
</tr>
<tr>
<td>Immunization</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
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Furthermore, none of these immunized animals got pregnant in the second year’s breeding season, while all control animals got pregnant and lambed at term (Table 3).

4. Discussion

Theoretically, immunocastration is a reversible phenomenon. The reversibility of immunocastration in natural processes, i.e., with gradual decrease in GnRH antibody concentrations over time, has been noted in several studies (Keeling and Crighton, 1984; Grieger et al., 1990; D’Occhio et al., 2001). However, several studies related to restoration of pituitary and ovarian functions using hormone treatment after immunocastration have been reported. Serum concentrations of LH in actively or passively immunized ewes were restored by continuous (hourly) administration of a GnRH agonist that did not cross-react with the antibodies (Adams and Adams, 1986; Herman and Adams, 1990; Sakurai et al., 1992). Also, restoration of spermatogenesis using eCG in GnRH immunized bulls has been achieved (Oatley et al., 2005). The use of eCG in estrous synchronization protocols in sheep is well established. Fourteen days progesterone impregnated sponges insertion accompanied with a single eCG injection at sponge withdrawal increases ovarian response and percentage of multiple births from induced ovulation (Pearce and Robinson, 1985). Additionally, GnRH treatment is known to induce ovulation in anestrous sheep using traditional synchronization programs (Bartlewski et al., 2001, 2004). In the present study neither eCG nor GnRH analogue injections combined with FGA (progestagen) treatment were able to induce estrous in immunized ewes.

GnRH injections to the non-cyclic immunized ewes in early ages (Brown et al., 1995) or adult ages (Jeffcoate et al., 1978) did not cause a marked increase in LH concentrations. It was concluded that immunization may have resulted in either reduced sensitivity of the pituitary gland to GnRH stimulation, a diminished capacity to produce and secrete LH and FSH or depleted pituitary pool of gonadotropins. In 35% of sexually mature bulls immunized against LHRH the testes continued to decrease in size for 4 months and did not show any re-initiation of growth for 1 year after immunization (D’Occhio et al., 2001). It was concluded, therefore, that active immunization against GnRH can induce a long-term, possibly permanent, suppression of reproductive function in bulls. Thus, a permanent castration like effect could be speculated for protein combination used in the present study as well.

The mechanism for sustained suppression of reproductive functions after immunization against GnRH is poorly understood. Preliminary findings in sheep and swine suggest that basal hypothalamus-median eminence is a primary target site for anti-GnRH antibodies, and immunization may lead to a disruption of the integrity of basal hypothalamus-median eminence (Molenaar et al., 1993; Clarke et al., 1998). Hernandez et al. (2005) reported vacuolized large basophil cells known as ‘castrate cells’ in histological examination in castrated rats’ pituitaries but not in the immunocastrated ones. Similarly, histological evaluation of anterior pituitaries of castrated
and GnRH immunized bulls and ram lambs did not indicate any pathological or morphological differences (unpublished results). It appears that there is little information to indicate that GnRH immunization can generate a permanent effect on the pituitary by causing pathological changes.

There are some reports that eCG induces some immune response and therefore eCG antibodies are generated (Bodin et al., 1997). In some estrous synchronization programs lower fertility rates were attributed to this fact. Animals in this study had been treated with eCG previously during the preceding years. So, in order to eliminate this possibility half of the animals in immunization group were treated with GnRH because GnRH treatment is known to induce ovulation in anestrous sheep (Bartlewski et al., 2001, 2004). Nevertheless, this approach did not contribute to restoration of reproductive functions.

The response of ovaries to eCG injection in immunized animals remains to be evaluated. eCG exerts its gonadotropic effect via FSH and LH receptors on the ovary (Murphy and Martinuk, 1991). Neither in the first attempt in eCG treated immunized group nor in the second and third attempts could induce ovarian functions in immunized animals. Mariana et al. (1998) were able to induce preovulatory follicles in ewes immunized against GnRH early in life by multiple injections of FSH, LH or hCG. Similarly, spermatogenesis has been restored in GnRH immunized bulls using eCG administered i.m., every 2 weeks for 80 days (Oatley et al., 2005). In the present study, immunization, possibly, caused the ovaries of ewes to regress to a pre-pubertal stage and the time and total dose of eCG applied in the present study were too short to recover from this stage. Alternatively, eCG could induce follicular developments on the ovary but preovulatory surge could not occur because of high level of circulating GnRH antibodies. This aspect requires further investigation.

Immunized animals did not get pregnant in the second year’s breeding season. GnRH antibody concentrations were still high during this time (Fig. 3). Prolonged immunocastration might be the result of high antibody concentrations.

There has been no dose response trial for GnRH fusion proteins in the ewes. In order to achieve a reversible or transient immunocastration it is important that immunization against GnRH should achieve a short-term direct immunoneutralization of GnRH by GnRH antibodies. If immunization disrupts the tissues in the median eminence associated with the presence of high levels of GnRH antibodies a long-term immunocastration is achieved, but this immunocastration will not be reversible.

5. Conclusion

There is a possibility that lower doses of these fusion proteins may be able to induce only short-term immunoneutralization of GnRH by GnRH antibodies and therefore allows restoration of reproductive functions upon request.

These results suggest that GnRH immunization exerts its effect via the hypothalano-pituitary axis and that more than such stimulation is required to overcome the reproductive suppression. Determining the way of response of ovaries to eCG injection in immunized animals requires further investigation.

Acknowledgements

This project was supported by Adnan Menderes University Scientific Research Projects Commission (Project no: ÇMYO-03001) and National Research Initiative Competitive Grant
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