Evaluation of one-shot vaccination protocol for suppressing reproductive functions in rams using encapsulated ovalbumin-LHRH-7 protein

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Received: 19-07-2016 Accepted: 20-02-2017 DOI: 10.18805/ijar.v0iOF.8472

ABSTRACT
The objective of study were to determine the effectiveness of Ovalbumin-LHRH-7 (OL) protein administered with cytosine guanine (CpG) adjuvant and Incomplete Freund’s Adjuvant (IFA), and used one-shot immunization (single-dose vaccination) protocol in which booster dose included in microspheres in rams. Fifty ram lambs at about a year old were used. Treatment groups receiving Ovalbumin LHRH and control contained 10 animals. They were stratified according to age (weeks), live weight and scrotal circumference size, and were randomly assigned to five groups. Scrotal circumference, sexual activities and the numbers of rams having sperm in the ejaculate were affected from treatment (P<0.05) depending on the dose and vaccination protocol. However, immunization did not affect live weight changes in any treatment groups (P>0.05). Findings clearly demonstrate that the effects of OL immunization on reproductive traits in yearling rams were prominent when it was administered at higher dose and classical one primary and one booster immunization as free protein form. Also we observed that the effect of higher and single dose of OL protein in encapsulated form on reproductive traits had the partial suppressing. CpG adjuvant along with IFA was proved to be an effective adjuvant and could be suggested to be used and alternative to FCA in hormone immunization.

Key words: LHRH immunization, Encapsulation, Single shot vaccination, Scrotal circumference, Sperm production, Ram.

INTRODUCTION
The typical OL immunization protocol is consists of a primary injection of antigen in a water-in-oil emulsion of Freund’s complete adjuvant (FCA), followed by one or two booster injections of antigen in Freund’s incomplete adjuvant (IFA). However, the inflammatory process induced by Freund’s adjuvant can cause skin lesions (Reeves et al., 1989; Bilskis and Sutkeviciene; Seghatoleslam et al., 2014). Thus, acceptance for any vaccine commercialization in pets as well as other food producing animals will require an alternative adjuvant because of this skin lesion of Freund’s. Potential use of bacterial cytosine guanine oligode oxynucleotides (CpG ODN) which are rich in CpG motifs in mammals as immunogenic substance in immunization protocols has been studied and suggested to be used as an alternative adjuvant for Freund’s (Bode et al., 2011). One potential advantage of CpG ODN over modified Freund’s is that CpG might cause less tissue damage. Use of a synthetic CpG (CpG ODN 2006) as an adjuvant in OL immunization protocol was demonstrated to be successful in inducing immune response and suppression of reproductive functions in rats (Conforti et al., 2007) and cattle (Conforti et al., 2008).

The objective of this study was to evaluate the effectiveness of two different doses of OL protein administered with CpG adjuvant instead of Freund’s in a classical one primary and one booster immunization protocol, and injected in a single-dose vaccination protocol (injecting with a mixture of free OL antigen and encapsulated OL antigen only once).

MATERIALS AND METHODS
Animals and treatments: Fifty native ram lambs at about a year old were used in this study. They were stratified according to age (weeks), live weight and scrotal circumference size, and were randomly assigned to groups.

All treatments and procedures involving animals were performed according to the International Guiding Principles for Biomedical Research Involving Animals as issued by the International Organizations of Medical Sciences.

Preparation of antigen and immunizations: OL protein was produced by recombinant DNA techniques previously described by Zhang et al. (1999). Recombinant OL gene was over-expressed in E. Coli. His-bind affinity chromatography with using a Ni2+ column allowed for purification of the proteins, and then they were suspended...
in 6 M urea. Incomplete Freund’s adjuvant and CpG DNA were used as adjuvants. Depending of the dose to be used in treatment groups, different amounts of OL fusion protein in free (neat) or encapsulated form was emulsified in CpG adjuvant and incomplete Freund’s adjuvant (Sigma, St Louis, MO, USA).

**Treatment and doses:** Treatment and doses for five treatment groups in this study (10 rams per group) were presented in the Table 1.

**Data collection:** Live weights in all groups were determined at one month intervals. Scrotal circumferences were measured, initially, at bi-weekly intervals for two months and thereafter at one month intervals for six months and then two and three months intervals. At breeding season when ram lambs were about 76 weeks (wks) of age sexual behavior tests were performed. For this purpose rams were placed in a lot with ewes in heat and observed for mounting behavior. During this period semen was collected via electroejaculator from rams and was evaluated for presence and concentration of sperm. A second sperm collection was performed just prior to terminating the study when rams were 128 wks age. Data collection was terminated at 74 wks after immunization.

Data analysis was performed using GLM procedure of SAS (Version 9.3. SAS Inst. Inc., Cary, NC, USA) for repeated measures to determine main effects of treatment, time and treatment x time for each of response variables (live weight and scrotal circumference). Reproductive behaviors data were analyzed using chi-square method. Sperm data were analyzed using GLM analysis. Duncan’s Multiple Range Test was used for pairwise comparisons. Data were presented as means ± SEM.

**RESULTS AND DISCUSSION**

Mean initial live weights (±SEM) in control and each treatment group were 36.20±1.70 (control), 34.26±1.80 (D1); 35.31±1.70 (D2); 37.08±1.70 (ED1); and 35.74±1.70 kg (ED2). Live weights increased steadily (Figure 1) in all groups until 80 wks of age, whereas there was a relatively sharp decrease happened when animals were 88 wks of age. Following the increase at 92 wks age, slightly small decreases continued in all animals until they reached at 108 wks of age. Thereafter live weight started to increase steadily through the end of study. The mean finishing weights (±SEM) in the control and treatment groups were 58.37±3.10 (control); 56.20±3.35 (D1); 58.47±3.35 (D2); 57.24±3.67 (ED1); and 64.12±3.67 kg (ED2). The differences among groups at any ages were not significant as statistical. In other words, there was no effect (P>0.05) of treatment group on live weight (Figure 1).

Mean initial scrotal circumference (±SEM) of control and treatment groups were presented in Figure 2. The mean initial scrotal circumference were respectively 23.50±1.07, 22.94±1.14, 23.17±1.07, 23.28±1.07 and 23.17±1.07 cm for control, D1, D2, ED1 and ED2 groups (Figure 1). The differences between groups for scrotal circumference in all groups were statistically not significant in the beginning of the study. Scrotal circumference in control group animals increased steadily until 76 wks of age and reached at average 31.00±1.89 cm. Because of the seasonal effect, however, a fluctuated decrease happened between average 26.81±1.53 cm and 24.75±1.59 cm until 108 wks of age in all groups. For the last four months of the study there was a sharp increase in scrotal circumference in this group.

Biological response to OL immunization was assessed from scrotal circumference measurements. Scrotal circumference was affected from treatment (P<0.05) depending on the dose and delivery systems. Although scrotal circumference started to decline in all treatment groups beginning at the 4th wks of first immunization, these differences from control values were not significant (P>0.05) until 8th wks of immunization (60 wks age). Thereafter, the differences among control and treatment groups were statistically significant until the end of study (P<0.05).

Group D1 differed only for 8 wks (between 60 and 68 wks age) than control group in this trait (P<0.05). The differences among D1, ED1 and ED2 groups were not significant for the most of the duration of the study (P>0.05).

Immunization affected scrotal circumference most dramatically in D2 group (P<0.05). Scrotal circumference in this group decreased throughout the study until 100 wks age and happened to be average 3 cm smaller at this age than that determined at the beginning of the study. Beginning

<table>
<thead>
<tr>
<th>Group</th>
<th>Primary</th>
<th>Dose</th>
<th>Booster</th>
<th>T. Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>CpG</td>
<td>None</td>
<td>Same</td>
<td>-</td>
</tr>
<tr>
<td>D1</td>
<td>OL protein + CpG + IFA</td>
<td>0.3</td>
<td>Same</td>
<td>0.3</td>
</tr>
<tr>
<td>D2</td>
<td>OL protein + CpG + IFA</td>
<td>0.6</td>
<td>Same</td>
<td>0.6</td>
</tr>
<tr>
<td>ED1</td>
<td>OL protein + CpG + IFA + (Encapsulated OL p)</td>
<td>0.3+(0.9)</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>ED2</td>
<td>OL protein + CpG + IFA + (Encapsulated OL p)</td>
<td>0.6+(1.8)</td>
<td>None</td>
<td>-</td>
</tr>
</tbody>
</table>

D1. Animals received 0.3 mg OL protein with CpG and IFA at the first immunization and were boosted with same vaccine four weeks later. D2. Animals received 0.6 mg OL protein with CpG and IFA at the first immunization and were boosted with same vaccine four weeks later. ED1. Animals received 0.3 mg OL protein with CpG and IFA together with 0.9 mg of encapsulated OL protein. ED2. Animals received 0.6 mg OL protein with CpG and IFA together with 1.8 mg of encapsulated OL protein.
at 64 wks age, scrotal circumference was significantly smaller in this group than that of control group till the end of study. Similarly, beginning at 80 wks age, scrotal circumference was significantly smaller in this group than that of D1 group till the end of study (P<0.05). On the other hand, the differences among D2, ED1 and ED2 groups were not significant for the most of the duration of the study (P>0.05). Except 100 wks age, scrotal circumference in ED1 group did not differ from control group for whole duration of the study (P>0.05). Group ED2 differed only for 4 wks (between 64 and 68 wks age) than control group in this trait (P<0.05). Differences among treatment groups were not significant for the most of the duration of the study (P<0.05).

Sexual behavior data in control and immunized rams were given in Figure 3. All rams in control group either had interest in female but did not mount/mate (1/8, 13%) or mated ewes (7/8, 87%). Sexual activities were affected from immunization in all treatment groups (P<0.05). D2 group had highest numbers (6/8, 75%) of rams which had not interest in female. Numbers of animals in each group which had interest in female but did not mount/mate ranged within 1-3. D2 group did not have any animals which had interest in female but did not mount/mate. Seven of 8 animals (87%) in control group exhibited mounting/mating activity whereas these numbers were observed as 4/8 (50%), 2/8 (25%), 1/7 (14%) and 1/8 (13%) for D1, D2, ED1 and ED2 groups, respectively. The differences between control and treatment groups were statistically significant (P<0.05).

Numbers (n) and percentage (%) of rams having sperm in ejaculate and sperm concentrations (10^9/ml) in control and immunized rams were given in Figure 4. First sperm collection was performed at 28th wk of immunization.
when rams were 76 wks old. Spermatozoa were found in ejaculates of all control animals (8/8, 100%), whereas 7 of 8 animals (88%) in D1; 3 of 7 animals (43%) in D2; 5 of 8 (63%) animals in ED1 and 6 of 7 animals (86%) in ED2 groups had spermatozoa in their ejaculates at this time. While differences between control and D2 and ED1 groups were significant (P<0.05) for this trait, differences between control and D1 and ED2 groups were not significant (P>0.05).

Second sperm collection was performed at 74th wk of immunization when ram lambs were 124 wks old. Spermatozoa were found in ejaculates of all control animals (6/6, 100%). 8 of 8 animals (100%) in D1; 3 of 6 animals (50%) in D2; 5 of 7 (71%) animals in ED1 and 6 of 6 animals (100%) in ED2 groups had spermatozoa in their ejaculates at this time. While differences between control and D2 and groups were significant (P<0.05) for this trait, differences between control and D1, ED1 and ED2 groups were not significant (P>0.05) at the second sperm collection.

The percentage of animals having sperm in the ejaculate in all immunized groups tended to increase in the second sperm collection however these differences were not significant (P>0.05). Sperm concentrations at the first collection ranged from 2.23x10⁹/ml to 3.42x10⁹/ml in all groups. The values with sperm concentration in first and second collection for all groups are presented in Figure 5. D1 group has the highest concentration at this time and was significantly different (P<0.05) from other groups. However, immunization did not reduce sperm concentration in any treatment groups (P>0.05). The lowest value (1.89x10⁹/ml) for sperm concentration at the second collection was determined for D2 group. Immunization did not affect sperm concentration in any treatment group (P>0.05) in the second sperm collection. The differences in sperm concentration between first and second collection in all groups were not significant (P>0.05).

Several researchers reported the suppression in testicular development in response to LHRH immunization by using various LHRH antigens (Kiyma et al., 2000; Ferro et al., 2004). Although testicular development was suppressed in all mentioned studies, there was an increase in scrotal circumference or testicular mass. In previous OL immunization studies in ram lambs (Ülker et al., 2009b) or buck kids (Ülker et al., 2009a) scrotal circumference either remained as almost the same size as measured at the beginning of the study or became slightly smaller. This kind of result was observed in this study only in D2 group.

A traditional, one primary and one booster, immunization was applied to D1 and D2 groups, whereas single injection protocol with encapsulated OL was applied ED1 and ED2 groups. D1 and ED2 groups responded to OL immunization similarly for only 8 and 4 wks, respectively, whereas the effect of immunization in D2 lasted longer (till the end of study). This might be attributed to the dose of OL antigen (0.6 mg) used in this group. This suppressive and long lasting effect without CFA supports the findings of Conforti et al. (2007, 2008) that CpG adjuvant could be easily used an alternative for CFA.

Scrotal circumference measurements in single shot immunization (ED1 and ED2) groups tended to be smaller than that of control values. However, these differences were not significant as statistical. So, single shot immunization with encapsulated OL protein using either dose seemed not to be effective for a long lasting testicular suppression. Age of rams at immunization might be one of the factors for this
low suppression. Active immunization against LHRH at earlier ages such as 3–4 wks (Brown et al., 1994), 15 wks (Kiyma et al., 2000), 20–21 wks (Brown et al., 1994), 18 wks (Ülker et al., 2001) or 10 wks of age (Ülker et al., 2005) suppressed testicular development in ram lambs. Similar results were obtained in previous studies in which ram lambs or buck kids were immunized against LHRH at 18-19 wk of ages using same OL protein (Ülker et al., 2009a,b). Immunization was performed at about a year old age in the presented study and this age was considerably a late age comparing the previous studies.

Sexual behavior trial was performed at 28th wk of immunization when rams were average 76 wks old. A considerably high percentage of animals in control group (87%) exhibited mounting/mating activity. These activities were suppressed in treatment groups (P<0.05). Mounting activity is learned under the presence of testosterone, and even when testosterone is decreased the mounting activity is still present. The findings of the present study are in agreement with previous studies that sexual behaviors decreased in physically or immunologically castrated animals (Brown et al., 1994; Godfrey et al., 1996; Ülker et al., 2005). It is a well-established phenomenon that castration causes reduction in sexual behavior due to lack of testosterone produced in the testes. The changes in castration induced sexual behaviors have been found to be highly variable, and usually a complete elimination has not been obtained. The failure of complete elimination of sexual behavior in castrate animals regardless of castration age (pre- or post-pubertal) indicates the presence of some functional aspects of central nervous system that affects sexual behavior independent of testosterone (Senger, 1997). Although it is suppressed, the presence of sexual activity in immunized animals in the present study coincides with this phenomenon. Therefore, it appears that sexual behavior alone is not a valuable sign for the reproductive status of an individual (Sarmah et al., 1997; Kerketta et al., 2014). Nevertheless, as observed in D2 groups, the more scrotal development suppressed in response to LHRH immunization the more sexual behavior suppressed.

LHRH immunization suppressed spermatogenesis in bulls (Robertson et al., 1982), goat bucks (Godfrey et al., 1996) and ram lambs (Ferro et al., 2004) however, complete elimination of spermatogenesis in all treated animals could not be achieved. In previous studies done with this OL protein it has been demonstrated that sperm production was completely eliminated for at least until 37 wks age (Ülker et al., 2005); 41 wks age (Ülker et al., 2009b) and 44 wks age (Ülker et al., 2009a) in all immunized animals. In the present study complete elimination of spermatogenesis could not be achieved in all animals in treatment groups.

When both scrotal circumference and sperm production as biological responses are considered it has been noted that D2 group responded to the immunization most effectively. LHRH antibodies could not be studied in this study and, therefore it is not known whether the immune response profile to the types of OL protein immunization worked as it was proposed, i.e., encapsulated OL protein was released gradually and generated a booster effect in ED1 and ED2 groups. The presence of partial suppressing effect of higher and single dose of OL protein in encapsulated form (ED2) on reproductive traits could be in indication for later gradually release to induce booster effect. Inulin and saponin are immunogenic substances and are used as adjuvants themselves. These were added in agarose bead and these inuline and saponin containing beads were emulsified with CpG and IFA in vaccine solution. It is not known if this kind of mixing could generate an antagonistic effect for inducing immune response in the present study.

Results demonstrate that single-dose vaccination protocol seemed not to work in this study. The effects of OL immunization on reproductive traits in yearling rams were prominent when it was administered at higher dose and classical one primary and one booster immunization as free protein form. Nevertheless, higher and single dose of OL protein in encapsulated form generated partially suppressing effect on reproductive traits. Age might be a factor for this...
weak response. CpG adjuvant along with IFA was proved to be an effective adjuvant and could be suggested to be used and alternative to FCA in hormone immunization. A future trial might be designed to test the effectiveness of OL protein in single injection protocols at earlier ages. In these protocols alternative use of immunogenic substance in generating beads and as adjuvants in vaccine emulsifying solutions might be tested as well.

ACKNOWLEDGEMENT

This study was supported by Yuzuncu Yil University Research Fund (Project no. 2007-ZF-B-023).

REFERENCES


