*Animal Science Papers and Reports* vol. 31 (2013) no. 3, 261-268
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# The use of encapsulated Ovalbumin-LHRH-7 (OL) protein in single-dose vaccination protocols for LHRH immunization

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*(Received October 1, 2012; accepted July 2, 2013)*

**The objective of this study was to evaluate effectiveness of Ovalbumin-LHRH-7 (OL) protein when injected in crude, purified, free or encapsulated forms and using a single vaccination protocol along with CpG, inulin and saponin adjuvants. Fifty six C57BL/6 mice in seven groups (n=8) received various treatments and doses: Group 1 was control; Group 2 and 3 were injected twice with purified or crude OL protein, respectively, 4 wks apart. Group 4 and 5 were injected only once with purified or crude OL protein, respectively. Group 6 was injected only once with a mix of purified OL protein and encapsulated purified OL protein. Group 7 was injected only once with a mix of crude OL protein and encapsulated crude OL protein. There was an immunization effect observed on the $I^{125}$ LHRH % binding ($P<0.05$). Antibodies (Abs) against LHRH were identified on week 5 of immunization in groups 2, 3 and 4. Boosting at week 5 caused a significant increase in LHRH antibody (Ab) concentrations in groups 2 and 3. Numbers of pregnant animals and prengnancy rates were suppressed in all treatment groups at various degrees ($P<0.05$). Numbers of pups born were affected by immunization ($P<0.05$). Concluding, immunization with OL protein generated either biological or both immunological and biological effects in the most of treatment groups. The study confirmed the earlier findings that purified OL protein with CpG adjuvant is effective in inducing immune response and suppressing reproductive functions. However, the original idea that the non-capsulated antigen/adjuvant mix would work as primary injection, while encapsulated counterpart would mimic booster injections in a single vaccination protocol could not be confirmed in this study. Further studies to determine affecting factors for single-injection LHRH immunization are needed.**

**KEY WORDS: encapsulation / immunization / LHRH / mice / reproduction**

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Immunizing farm animals against luteinizing hormone releasing hormone (LHRH) has been studied as an alternative for sterilization technique (immunocastration) for surgical castration [Reeves *et al*. 1989, Bonneau and Enright 1995, Thompson 2000]. A recombinant LHRH fusion protein Ovalbumin-LHRH-7 (OL) was reported by Zhang *et al.* [1999]. This fusion protein has been evaluated for its effectiveness in suppression reproductive functions in several species and found to produce satisfactory results in these species in terms of anti-LHRH antibody production and sterilization [Geary *et al*. 2006, 2011, Conforti *et al*. 2007, Ülker *et al*. 2009ab].

Although OL protein was determined to be effective in various species, several things regarding the mentioned protein need to be optimized to use it more effectively in LHRH immunizations. These things are: purifiying, adjuvant(s), dose and delivery systems (single *vs.* one primary and one or more booster injections) – related issues.

OL protein produced is insoluble and needs to be solublized before purification. Purification is expensive and time consuming process. Besides, insoluble proteins made good antigens possibly because of their persistence in the immunized animal [Harlow and Lane, 1988]. So, if OL protein can be used without purification (as crude protein) this migt eliminate all purification procedure, save time and reduce production cost.

The number of immunization necessary for successful fertility control is an important factor to be considered in animal immunized against native hormones. Producers or local authorities trying to control wild or feral populations would benefit from an effective, single-dose vaccine in terms of management easiness, reducing additional costs associated with time, labor and the cost of each dose itself. Ideally, a single-dose contraceptive vaccine would be effective in controlling the size of population even if each treated individual was immunized only once in its lifetime. The number of injections necessary for desired vaccine effect and longevity is influenced by type of delivery system. Single-dose vaccines usually require a delivery system that releases antigen and adjuvant in a slow manner in order to maintain relatively high levels of immunogens in the system for a prolonged period of time. The idea here is that the slow antigenic release would stimulate the effects of booster injections thus eliminating the need for booster injections. One of the most frequently used delivery systems for slow, controlled antigenic release is antigen encapsulation in polymers. The lactide:glycolide ratio dictates the rate at which the antigen is released into the system because each polymer has a certain degradation rate. This type of delivery system is commonly used in single-dose vaccines and has been tested in immunocontraception studies [Turner *et al*. 1996, Kirkpatrick *et al*. 1997]. Potential use of OL protein antigen encapsulated in polymers for LHRH immunization in a single-dose vaccine protocol has not been tested.

Taken together, it is desirable to test various forms of OL protein using different delivery systems to improve the effectiveness of the protein. The objective of this study was to evaluate effectiveness of OL protein when injected in various forms and using a controlled release technology as a means of achieving long-lasting antibody responses *via* a single inoculation.

## Material and methods

### Preparation of antigen

The plasmid used to transform *E. coli* cells contained a fragment of the carrier protein ovalbumin with seven inserts of the LHRH sequence [Zhang *et al*. 1999]. The resulting fusion protein Ovalbumin-LHRH-7 (OL), is approximately 55 kDa in size and was insoluble (crude). For purified protein OL was solubilized in 6.5M guanidine and purified using nickel chelation chromatography. A protein assay (BCATM Protein Assay Kit, PIERCE) was performed to determine the concentration of OL in urea.

### Treatment groups, vaccine preparation and immunizations

Fifty six C57BL/6 mice (8-16 weeks old) were used. They were stratified according to age and randomly assigned to one of the vaccine treatments. Food (mice chow) and water were provided *ad lib*. Animals were kept in Washington State University Experimental Animal Laboratory Building. All procedures related to animal experimentation met the International Guiding Principles for Biomedical Research Involving Animals as issued by the International Organizations of Medical Sciences.

Seven groups each containing 8 mice (4 in each cage) received the treatments and doses described in Table 1.

**Table 1**. Treatment groups, imunizations (primary and booster) and doses of used OL protein (μg)

| Group | Primary | Dose | Booster | Dose | T. dose |
|---|---|---|---|---|---|
| Control | CpG | none | same | - | - |
| 2 | Free purified OL protein | 20 | same | 20 | 40 |
| 3 | Free crude OL protein | 20 | same | 20 | 40 |
| 4 | Free purified OL protein | 20 | none | - | 20 |
| 5 | Free crude OL protein | 20 | none | - | 20 |
| 6 | Free purified OL protein + (purified OL protein in agarose, innulin and saponin coated bead) | 20+(4) | none | - | 24 |
| 7 | Free crude OL protein + (crude OL protein in agarose, innulin and saponin coated bead) | 20+(6) | none | - | 26 |

Briefly, Group 1 was control group injected with only CpG adjuvant.
Group 2 was injected with free purified OL protein in CpG adjuvant (wk 0).
Group 3 was injected with free crude OL protein in CpG adjuvant (wk 0).
Groups 2 and 3 received booster injections 4 wk later as in the first injection.
Group 4 was injected with free purified OL protein in CpG adjuvant (wk 0).
Group 5 was injected with free crude OL protein in CpG adjuvant (wk 0). Groups 4 and 5 received single injection. No booster was given.

Group 6 was injected with a mix of free purified OL protein with CpG adjuvant and encapsulated purified OL protein (wk 0). Purified OL protein was embedded in the capsule made of agarose, inulin and saponin.

Group 7 was injected with a mix of crude free OL protein with CpG adjuvant and encapsulated crude OL protein (wk 0). Crude OL protein was embedded in the capsule made of agarose, inulin and saponin. Groups 6 and 7 received single injection. No booster was given. The idea was that the non-capsulated antigen/adjuvant mix would work as primary injection, while encapsulated counterpart would mimic booster injections.

Two types of bead for encapsulation were prepared: agarose-innulin-saponin mixture coating bead and OL protein embedded (encapsulated) in this bead and agarose bead was generated to have OL protein embedded in agarose-inulin-saponin bead. Agarose (quilaja bark, SIGMA) comprised either 6% or 4% of the bead. Inulin (dahlia tubers, SIGMA) and Saponin (quilaja bark, SIGMA) comprised 5% and 4% of the bead content, respectively.

The ova-LHRH fusion proteins used in all immunizations originated from the same batch. All immunizations were done with CpG adjuvant. Freund's Incomplete Adjuvant (85% light mineral oil NF (Drakeol® 5; Penreco, Dickinson, TX) and 15% mannide monooleate (SIGMA, St. Louis, MO) was used in CpG adjuvant formulation. The CpG DNA used in this study was the nuclease-resistant phosphorothioate CpG ODN 2006 (Oligos Etc) presenting the following nucleotide sequence: TCGTCGTTTTGTCGTTTTGTCGTT [Zhang *et al*. 2003].

Vaccine preparations were repeatedly mixed between two syringes with a double hub connector until emulsification. Total volume per animal was 200 µl. The primary injection and the boosters were given (IM and SC) on alternate sides at the base of the tail and at the nape of the neck.

#### Pregnancy trials

To determine the biological response to OL immunization two pregnancy trials were performed. In the first trial, males were introduced to the cages on wk 9 and removed on wk 14. All mice having pups in the first trial were weaned, and males were let inside on wk 16 for second trial. The males were removed on wk 18. During both pregnancy trials males were switched among cages to eliminate male-related infertility risk. Pups' numbers were recorded in both pregnancies.

#### Blood sampling

Blood was withdrawn on week 5, 9, 13 and 17 from the first immunization (wk 0). Approximately 25 µl of blood was withdrawn from the saphenous vein into heparinized microtubules and transferred into 120 µl heamaglunation buffer. Blood samples were centrifuged in a microcentrifuge (16,000×g, 20 min, 4°C), and sera were separated and kept frozen at -20°C until analysed.

**Assessment of anti-LHRH antibody production**

Anti-LHRH antibody production was assessed by radioimmunoassay. Serum antibody binding activity was measured by the amount of $^{125}$I-LHRH bound in 1:1,000 diluted sera [Johnson *et al*. 1988].

**Statistical**

The effects of treatment (group) on the percentage of anti-LHRH antibody binding activity ($^{125}$I-LHRH % binding) and reproductive measurements were analysed by analysis of variance (ANOVA) using the general linear models procedure (Proc. GLM) of SAS Software (Version 9.1). Values are expressed as means±SEM. The level of significance was set at $P<0.05$.

## Results and discussion

Sera from blood samples collected at weeks 5, 9, 13 and 17 were assayed for LHRH antibody activity. There was a protein effect on the $I^{125}$ LHRH % binding ($P<0.05$). Abs against LHRH were present on week 5 of immunization in groups 2, 3 and 4. Boosting on week 5 caused a significant increase in LHRH Ab concentrations in groups 2 and 3 ($P<0.05$). A slight decrease in LHRH Ab concentration was observed in group 4 over 12 weeks. No LHRH Ab production was identified in other treatment groups (Fig. 1).

Data regarding the reproductive traits are presented in Table 2. Numbers of pregnant animals and pregnancy rates were suppressed in all treatment groups at various degrees ($P<0.05$), except group 7. While there were no pregnant mice (0%) in group 2 in the first pregnancy trial, pregnancy ratios were 25, 38, 50, 25 and 75% in groups 3-7, respectively. Smilar trend was observed during the second pregnancy trial.

Numbers of pups born were affected by immunization ($P<0.05$) except group 7. While mice of group 2 delivered no pups, the other treatment groups had lower mean pups numbers compared to control group. Litter sizes were calculated for only littering mice. With this respect, while litter size was 0 in group 2, regardless of treatment, pups numbers per mouse were smilar in all groups.

Immunization with purified or crude OL protein using classical one primary and booster injections with CpG adjuvant (groups 2 and 3) resulted in higher ($P<0.05$) $I^{125}$ LHRH % binding compared to other groups (Fig. 1). In group 2, both immunological (Ab production) and biological (suppressing reproductive functions) responses to OL immunization were numerically the highest. Pregnancy was suppressed completely in this group. Similar effect was observed in groups 3, 4 and 6, however, pregnancy measurements were numerically lower in these groups than in group 2. These findings are in accordance with those reporting effective immune and biological responses obtained using OL protein with CpG adjuvant in different species [Conforti *et al*. 2007, 2008].

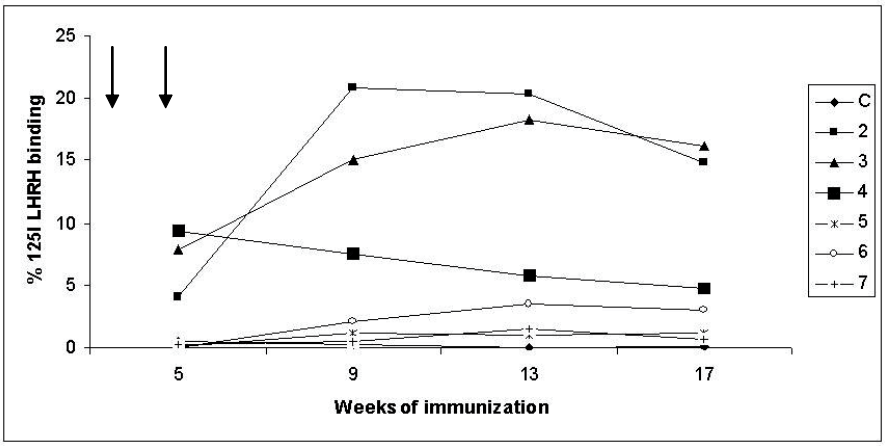


Fig. 1. Mean antibody binding to luteinizing hormone releasing hormone (LHRH) expressed as a percentage bound $I^{125}$ LHRH at 1:1,000 dilution in control and immunized mice. Arrows represent the time of immunizations.

**Table 2**. Reproductive indicators in control and immunized mice (n=8)

| Group | Pregnant animals | | Pregnancy rate | | Pubs born | | Litter size* | |
|---|---|---|---|---|---|---|---|---|
| | 1st mate | 2nd mate | 1st mate | 2nd mate | 1st mate | 2nd mate | 1st mate | 2nd mate |
| Control | $8^{a}$ | $7^{a}$ | $100^{a}$ | $71^{a}$ | $54^{a}$ | $39^{a}$ | $6.8^{a}$ | $5.6^{a}$ |
| 2 | $0^{c}$ | $0^{c}$ | $0^{c}$ | $0^{c}$ | $0^{c}$ | $0^{c}$ | $0.0^{b}$ | $0.0^{b}$ |
| 3 | $2^{bc}$ | $3^{bc}$ | $25^{bc}$ | $38^{bc}$ | $12^{bc}$ | $18^{bc}$ | $6.0^{a}$ | $6.0^{a}$ |
| 4 | $3^{bc}$ | $3^{bc}$ | $38^{bc}$ | $38^{bc}$ | $15^{bc}$ | $22^{bc}$ | $5.0^{a}$ | $7.3^{a}$ |
| 5 | $4^{b}$ | $4^{b}$ | $50^{b}$ | $50^{b}$ | $24^{b}$ | $32^{b}$ | $6.0^{a}$ | $8.0^{a}$ |
| 6 | $2^{bc}$ | $2^{bc}$ | $25^{bc}$ | $25^{bc}$ | $13^{bc}$ | $12^{bc}$ | $6.5^{a}$ | $6.0^{a}$ |
| 7 | $6^{ab}$ | $4^{b}$ | $75^{ab}$ | $57^{ab}$ | $39^{ab}$ | $31^{ab}$ | $6.5^{a}$ | $7.8^{a}$ |

*Only littering mice' pups numbers were used in calculating litter size.
[abc]Within the column means bearing different superscript letters are significantly different at $P<0.05$.

None of the mice of group 2 had pups born, while the other treatments' groups included both littered mice and mice that did not litter. Means for immunized groups had lower pups numbers compared to control group ($P<0.05$) except group 7. Nevertheless, when litter sizes were calculated for littering mice only it was determined that, regardles of treatment, pups numbers per mouse occurred smilar in all groups (Tab. 2). Apparently, immunization did not reduce the offspring numbers in mice *per se*, instead, it suppressed reproduction completely in responding animals. In this study reproductive organs were not examined, however, smilar findings such as degeneration of the ovaries and uteri [Wang *et al*. 2010] or suppression of folliculogenesis [Khan *et al*. 2008] in the LHRH vaccinated female mice were reported by various authors.

In all treatments, purification seemed to generate positive effect upon OL protein: in group 2 and 4, Ab production was higer also in these groups and group 6 pregnant mice numbers were lower than in groups in which crude protein was used, except

group 3. Immunization with crude (insoluble) OL protein using a primary and a booster shot (group 3) induced Ab production and caused suppression in reproductive traits. Although mean differences in pregnancy indicators were not significantly different from that of immunization with purified protein (group 2) this crude OL protein did not induce LHRH Ab production in some animals and consequently these mice which did not produce Abs got pregnant. Besides, a mouse which had considerably high LHRH Abs got pregnant in this group as well. In fact, immunizing with crude OL protein was expected to induce better immune response because of their persistence in the immunized animals [Harlow and Lane 1988]. This lesser effect of crude OL protein in inducing immunological and biological effect warrants further studies.

Response to booster immunization as an increased Ab production was observed in groups 2 and 3. Similar response was hypothesized to be seen in group 6 and 7 as encapsulated OL protein was expected to be released and would mimic booster injection. However, this kind of response was not noticed in this study. At this point, it is hard to know whether agarose bead encapsulating the protein did not dissolve any or at appropriate time or the amount of protein encapsulated was not enough to generate a boosting effect.

In conclusion, immunization with OL protein generated either biological or both biological and immunological effects in the most of treatment groups. This study confirmed earlier findings that purified OL protein with CpG adjuvant is effective in inducing immune response and suppressing reproductive functions. However, the original idea that the non-capsulated antigen/adjuvant mix would work as primary injection, while encapsulated counterpart would mimic booster injections in a single vaccination protocol could not be confirmed to work in this study. Further studies to determine affecting factors for single injection LHRH immunization are needed.

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