A TEST OF A REDUCED DOSE OF PORCINE ZONA PELLUCIDA VACCINE IN WILD HORSES

A RESEARCH PROPOSAL SUBMITTED TO THE BUREAU OF LAND MANAGEMENT

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RATIONALE FOR THE STUDY

Background

Currently a dose of 100 µg of porcine zona pellucida (PZP) is utilized to treat wild horses (*Equus caballus*) (Kirkpatrick and Turner 2002), 115 species of zoo animals (Frisbie and Kirkpatrick, 1998), and white-tailed deer (*Odocoileus virginianus*) (Naugle et al. 2002; Rutberg et al. 2004). The rationale for this dose comes from the original research of Liu et al. (1989), and subsequent research with wild horses (Kirkpatrick et al. 1990, 1991, 1992, and 1995) in which a dose of 65 µg was used successfully in wild horses. It was later determined that this 65 g dose was in reality closer to 100 µg and the discrepancy was in the protein quantitative analysis methodology and specifically in the protein standard utilized in the assay.

The same dose of 100 µg has been used successfully in a variety of species ranging in size from muntjac deer (*Muntiacus reevesi*, 14-35 kg) (Kirkpatrick et al. 1996a) to bison (*Bison bison*, 350 -1200 kg) (Kirkpatrick et al. 1996b). There are however, few studies that examine the efficacy of the PZP vaccine at other doses of PZP. In horses, 200 µg (Willis et al. 1994) and 400 µg PZP (Stafford et al. 2001) doses have been used with either equal success to the 100 µg doses (Willis et al. 1994) or less success (Stafford et al. 2001). Confounding these studies, and the ability to compare doses are the uses of differing delivering systems and adjuvants.

In white-tailed deer doses of 300 – 500 µg PZP have been used to achieve contraception with equal efficacy as the 100 µg dose (Miller et al. 2000), using the same adjuvants. In African elephants (*Loxodonta africana*) 600 µg PZP doses were successful in causing contraception (Fayrer-Hosken et al. 2000), but subsequent trials using doses of 400 µg (Delsink et al. 2002) and even more recent trials with 200 µg (Delsink, pers. comm..) were equally successful.

Another factor confounding evaluations of differing doses of any immunological approach to fertility control – or disease prevention for that matter – is the matter of

individual variation in response to a vaccine. It is clear from a number of studies, with a variety of species, that there are clear individual differences in response to an immunological challenge. This has been seen in horses (Liu et al. 1989; Turner et al. 2002), deer (Turner et al. 1996), elk (*Cervus elaphus*) (Garrott et al. 1998), and fallow deer (Cervus dama) (Deigert et al. 2003) and other captive exotic species (Kirkpatrick et al. 1996a;). Based on antibody titers it is clear that animals fall into groups of "normal" immune responders, poor responders and exceptional responders and these individual differences are probably the cause for the < 100% efficacy we see in almost all studies.

In only one species has any attempt been made to determine a "contraceptive threshold" for the PZP vaccine. Liu et al. (1989) suggested that anti-PZP antibody titers at or above the 60% level provided effective contraception, and subsequent studies have confirmed that approximate figure (Turner et al. 2002).

Economics

It is of interest to investigate the possibility of reaching contraceptive thresholds with smaller doses of immunogens, particularly for economic reasons. The production of PZP for use in wild horses is confined to very few laboratories and requires native PZP, as opposed to easily manufactured recombinant or synthetic forms of the vaccine. To date, no effective synthetic or recombinant ZP has been achieved, thus we remain reliant upon the native form of the antigen, extracted from porcine ovaries.

The current best available technology for producing the native form of PZP is at best what we refer to a "bench top" chemistry, which relies upon careful manipulation of small quantities of tissue at one time. Attempts at scaling up production will come at a cost of quality control, and the end-result is a product that requires a labor intensive effort. Because of federal regulations related to the Food and Drug Administration, the vaccine cannot be produced for a profit, thus it is provided at a cost of production, which is currently about $20/100 µg dose. These costs are reasonable until the PZP is incorporated into long-acting, one-inoculation formats,, such as the lactide-glycolide pellets currently in use in population level trials.

At the present time, the pellet technology includes two methods for manufacture. The first is the "hot extruded" method which requires about 950 µg per treatment, or a cost of just less than $200 for the PZP content, without pellet manufacturing costs. The problem here is that there is somewhere between 30% to 50% wastage in the pellet manufacture process, which makes this approach expensive for the BLM and disheartening at best, for the technologist manufacturing the vaccine. The second approach is the "cold evaporated" process, which uses about 450 µg PZP per treatment. This approach is being tested at the present time in the McCullough Peaks horses. The cold evaporated pellet would cost the BLM about $90, and the reduction in PZP amount and cost comes from a process that is close to 100% efficient, without wastage of the antigen. At the current time, and pending the outcome of the McCullough Peaks study, we are assuming that this will be the primary approach to PZP immunocontraception in wild horses in the future.

Expanding these cost projections to 1,000 horses, treatment with the hot extruded pellets plus a primer PZP dose would be about $220,000, and treatment with the cold evaporated pellets would cost about $110,000.

If a dose of 50 µg PZP, instead of the 100 µg PZP, would result in effective contraception, the costs could be further reduced to $110 for a hot extruded pellet and $110,000 for 1,000 horses, and $55 for a cold evaporated pellet and $55,000 for 1,000 horses.

Thus, the rationale for this study transcends pure science and the search for an immunological threshold. It is aimed directly at reduction in the cost of treating wild horses for the managing agency. The objectives of this proposed trial are to:

1) implement a controlled pilot study with captive wild horses, in an effort to evaluate antibody titer response to 50 µg PZP vaccine doses;

2) Specifically provide a primer PZP vaccine dose of 50 µg PZP + Freund's Modified adjuvant to 10 captive wild horses and a subsequent booster of 50 µg PZP + Freund's Incomplete adjuvant 3-4 weeks later.

3) Specifically draw pre-treatment blood samples and monthly post-treatment samples over 12 months, by jugular venipuncture. All serum harvested will be sent to the Science and Conservation Center, Billings, MT, for anti-PZP antibody titers analysis.

METHODS

PZP preparation

The native PZP antigen will be prepared at the Science and Conservation Center (SCC) by the modified method of Dunbar et al (1980). The antigen is screened for pathogenic bacteria and a PAGE gel permanent record of the batch is kept on file at the SCC. The antigen is stored at – 43 o C, titrated to 50 µg doses and transported frozen to the site of use.

Experimental Animals

Ten adult mares from the Palomino Valley wild horse holding facility will be separated and identified for this study. All mares will be treated with 50 µg PZP + 0.5 cc Freund's Modified adjuvant, by im injection in the hip or gluteal muscles. Three to four weeks later each mare will be treated with 50 µg PZP + 0.5 cc Freund's Incomplete adjuvant as described. For both the primer inoculation and the booster inoculation, the PZP antigen and adjuvant will be emulsified as described by Kirkpatrick et al. (1990).

Control Animals

Controls for this study will include 8 animals previously treated with 100 µg PZP + Freund's Modified adjuvant, followed by 100 µg PZP + Freund's Incomplete adjuvant, in the course of the recently completed adjuvant trial. Antibody titers for these 8 animals have already been determined and will be used as the comparison for the reduced dose treatments described above. Initial treatment of animals will be conducted by a representative of the SCC research team; booster inoculation will be given by a BLM employee or personnel designated by the SCC.

Antibody Titer Analysis

Pre-treatment and subsequent monthly blood samples will be drawn by jugular venipuncture from each mare, the serum harvested and sent to the SCC. Anti-PZP antibody titers will be measure4d by the method described by Bynum (2000). Test sera will be assayed in duplicate and expressed as a percent of positive serum standard, which consists of a pool of sera from horses that had previously been treated and demonstrated anti-PZP titers in the high-positive range (mean of experimental serum absorbance/mean of reference serum absorbance) and which had not become pregnant following treatment. Determinations used in these determinations correspond to the dilution of the reference sera giving 50% maximum binding. The same reference standard used for the controls, previously, will be used for the treated animals. Differences in antibody titers over time will be tested for significance by the Tukey-Kramer Multiple Comparison test and by unpaired t-test with Welsch's correction applied (Motulsky et al. 2001). All blood samples will be collected and prepared by a BLM employee.

Materials Needed

20 50µg doses of PZP vaccine
Three 5.0 glass syringes
2 Plastic Luer-Loc connectors for syringes
1 vial (10 cc) of Frfeund's Modified adjuvant
1 vial (10 cc) of freund's Incomplete adjuvant
Assorted needles
Centrifuge
120 Corvac sterile blood separation tubes
120 glass serum storage tubes
Permanent glass marker

Procedures

Vaccine storage: Keep vaccine frozen until the day of use and pack in cold packs during use. Thaw at the time of use. If it thaws before you use it, that's okay as long as it is kept cold.

Mixing PZP-Adjuvant Emulsion

Using a glass syringe and 18 g. needle, draw up 0.5 cc of adjuvant. Being careful not to lose the adjuvant, use the same syringe and needle to draw up the 0.5 cc of PZP. Connect the syringe with the adjuvant-PZP mixture to the second glass syringe using the Luer-Loc connector. Give approximately 100 strokes, pushing the mixture back and forth between the two glass syringes, creating a thick milky emulsion. Make sure all the emulsion is in one syringe, disconnect it from the empty syringe (keeping the Luer-Loc connector on the syringe with the emulsion) and connect it to an empty plastic 3.0 cc syringe. Empty, by pushing gently, the contents of the glass syringe into the plastic syringe. You may have to pull back gently on the plastic syringe plunger at the same time you are pushing on the glass plunger. Put an 18 g., 1.5 inch needle on the plastic syringe and inject into the gluteal muscle of the mare.

Repeat the procedure regardless of which adjuvant is used: you may wish to load several syringes at a time.

You do not have to wash syringes between making emulsions.

BUDGET

22 50 µg doses of PZP + Fed Ex shipping	$ 270
Three glass syringes (already available and supplied by BLM)	-
2 Plastic Luer-Loc connectors (as above)	-
1 vial Freund's Modified adjuvant	$ 20
1 vial Freund's Incomplete adjuvant	$ 20
Centrifuge (already available and supplied by BLM)	-
Blood collection and storage tubes (available and supplied by BLM)	-
Antibody titer analysis (120 samples at $20/sample)	$2,400
Travel/subsistant costs for SCC representative for initial treatment	$1,000
Total Cost	$$3,710*

* exclusive of BLM-supplied materials outlined above and horse maintenance

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