Use of an immunocontraceptive vaccine in feral Kaimanawa mares

K J Stafford, E O Minot, W L Linklater, E Z Cameron, S E Todd
Institute of Veterinary Animal and Biomedical Sciences and Ecology Group
Institute of Natural Resources
Private Bag 11-222
Massey University
Palmerston North

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Abstract

Porcine zona pellucida vaccine sourced from the USA caused a significant serological response in captive Kaimanawa mares. Two injections of the vaccine given two weeks apart gave a serological response similar to that resulting from three injections given every two weeks over four weeks. Two injections of a vaccine produced at Massey University given to a thoroughbred mare gave a similar response to that produced by the American vaccine. A serological response of this level is likely to prevent conception during the following breeding season. The mass production of the vaccine using porcine zona pellucida material is difficult in New Zealand due to the large number of sows' ovaries required per vaccine (around 80 ovaries per dose) and the limited number of sows killed at any meat plant. The American vaccine is a suspension which must be shaken vigorously before use. Moreover the material quickly settles out of suspension, and this may make it difficult to use in a dart gun under field conditions.

1. Introduction

Immunocontraception uses an animal's immune response to disrupt reproductive function by acting on specific molecules in the reproductive process. An injection of antigens (a vaccine) given to the target animal initiates production of antibodies against some molecule requisite to successful reproduction (Kirkpatrick & Turner 1991). Successful immunocontraceptive agents must prevent fertilisation without compromising the health of the individual exposed to immunisation (Castle & Dean 1996). One mechanism for inhibiting fertilisation involves raising antibodies against the ovum protein receptors for sperm (Kirkpatrick & Turner 1991).

The mammalian oocyte is surrounded by the zona pellucida through which the sperm must penetrate during the initial stages of fertilisation (Dunbar & Raynor 1980). It has been demonstrated that both active and passive immunisation strategies that target individual zona proteins can prevent fertilisation (Castle & Dean 1996). Injections of raw zona pellucida protein cause the female to raise antibodies against the sperm receptor on the ovum (ZP3) and inhibit fertilisation. Porcine zona pellucida have been found to be an effective inhibitor of fertilisation in a variety of species (Kirkpatrick &Turner 1991). Mouse and human zona pellucida are composed of three glycoproteins, ZP1, ZP2 and ZP3 (Castle & Dean 1996), and pig zona pellucida glycoproteins were found to be homologous to these (Hedrick 1996). Porcine zona pellucida vaccines are used to cause immunocontraception in horses because up to 200 eggs can be obtained from the ovary of a sow in contrast to the 3 or 4 obtained from a mare's ovary.

The contraceptive effect in horses was found to be correlated with high anti-pig zona pellucida concentrations (Liu et al. 1989). This effect persisted for

at least eight months and diminished as antibody levels declined (Liu et al., 1989). After one innoculation, a return to fertility was found to occur in the second year after vaccination (Turner et al. 1997). The efficacy of immunocontraception in horses is much increased by the use of two innoculations (90% -100%) compared with only one innoculation (efficacy of 19% - 28%) (Kirkpatrick et al. 1996). The first innoculation causes antigen recognition and a temporary increase in antibody titres. The second innoculation causes increased titres which last several months, and each subsequent innoculation increases the duration of high titres (Kirkpatrick et al. 1990). Thus a single booster innoculation is capable of maintaining contraception.

Seven consecutive years of porcine zona pellucida treatment of mares in the wild had no detectable debilitating side effects other than some ovulation failure and depressed urinary oestrogen concentrations (Kirkpatrick et al. 1996). Porcine zona pellucida immunisation can be applied to pregnant mares without interfering with current pregnancies or with the health of their foals. It does not affect social behaviours and is reversible (Kirkpatrick et al. 1990).

Vaccination protocols usually consist of two initial innoculations given over a 4 - 6 week period, followed by a booster vaccination approximately 9 months later (Liu et al. 1989). The most common route of delivery of porcine zona pellucida vaccine is by intramuscular injection. This method is suitable in captive animals that can be handled, but not free-roaming wild animals (Kirkpatrick & Turner 1991). Wild animals are innoculated by remote delivery of the vaccine using darts fired from a dart gun or pellets such as a biobullet fired from an air-rifle (Kirkpatrick et al. 1990; Willis et al. 1994).

In a previous report Stafford et al. (1998) reported on the failure of a porcine zona pellucida (PZP) immunocontraceptive vaccine administered in a biobullet to prevent conception in wild free-running Kaimanawa mares. The question arose as to whether these mares were capable of responding serologically to the PZP vaccine. The PZP vaccine has been reported not to prevent conception in other species, including white-tailed deer (Peck & Stahl 1997). This report details the serological response of Kaimanawa mares to the injection of a PZP vaccine sourced from the USA and on the response of thoroughbred mares to PZP vaccine produced locally.

2. Serological responses of captive Kaimanawa mares to PZP vaccine

2.1 SELECTION OF MARES

The 24 mares used in this study were adult animals captured during the 1997 muster and held initially at a farm near Ohingati and later at the property of Mr Lewis Wheeler of the Kaimanawa Wild Horse Preservation Society near

Hamilton. The mares were grazed together in one group and did not have a stallion running with them. They were held in paddocks with deer fencing.

The mares were identified by a paint brand and the mane was clipped to identify the treatment. The mares were freeze branded on 17 October for long-term identification. During blood sampling and vaccination the mares were restrained in a portable set of yards with a cattle crush.

2.2 TREATMENT

The mares were randomly allocated to one of three treatments ;

Control - eight mares were managed in an identical manner and restrained and handled as in the other two treatments but they were not vaccinated.

Double vaccine - eight mares were given two doses of the vaccine, the first on 30 July 1998 and the second two weeks later on 13 August.

Triple vaccine - eight mares were given three doses of vaccine, the first on 30 July, the second on 13 August and the third on 27 August.

2.3 VACCINE

The vaccine was obtained from Dr Richard Fayrer-Hosken at the College of Veterinary Medicine, University of Georgia, Athens, Georgia, USA. It was a suspension vaccine which had to be shaken vigorously before being administered by intramuscular injection into the rump. The solid portion of the vaccine settled out of the liquid quite rapidly.

2.4 BLOOD SAMPLING

A blood sample was taken from each mare into a 10 ml plain vacutainer by jugular venepuncture on four occasions (30 July, 13 August, 27 August and 17 October) before the animals were vaccinated when appropriate. The blood samples were placed on ice immediately after being taken and the serum centrifuged off at 3000 rpm for 10 minutes within 24 hours. The serum samples were divided into two aliquots and frozen at -2 °C until dispatched to Dr Richard Fayrer-Hosken in Georgia for analysis.

2.5 SERUM AND DATA ANALYSIS

The serum anti-zona pellucida IgG levels were measured using an enzyme-linked immunosorbent assay (ELISA) as described by Willis et al. (1994).

The changes in anti-zona pellucida IgG levels in the three treatments were analysed by repeat measures analysis of variance. The levels were compared across groups using a one-way analysis of variance.

2.6 SEROLOGICAL RESPONSE TO THE VACCINE

The serum anti-zona pellucida IgG levels were low in the blood sample taken from the mares on 30 July immediately before the initial vaccine was administered (Figure 1,Table 1). There was no significant difference in the IgG levels of the three treatments at this time.

The serum anti-zona pellucida IgG levels did not change significantly in the control mares throughout the experiment (Figure 1, Table 1). In both the vaccinated groups the serum anti-zona pellucida IgG level was not significantly greater two weeks after the first vaccine (13 August) but increased significantly ($P < 0.05$) after the second vaccination (Figure 1,Table 1). There was no significant difference between the levels at the third and fourth sampling, and the third vaccination did not increase the level significantly. The difference between the IgG levels of the control group and the group receiving 3 vaccines was significant ($P < 0.05$) on the 27 August and 17 October (Figure 1). The difference between the IgG levels of the control group and the group receiving two vaccines was significant ($P < 0.05$) on 17 October (Figure 1).

2.7 DISCUSSION

There was a substantial serological response to vaccination with a PZP immunocontraceptive vaccine in captive Kaimanawa mares. The response varied greatly between individual mares with some of the vaccinated mares having a very small increase in IgG levels or none. The overall mean response to both the two vaccine and the three vaccine regimes was likely to be sufficient to prevent conception in the following breeding season (Fayrer-Hosken, pers. comm.).

The reason for the variation in response is unknown but has been observed and commented upon in similar studies elsewhere (Liu et al. 1989; Willis et al. 1994). This variation in serological response is likely to influence the contraceptive efficacy of the vaccine regime (Willis et al. 1994).

Some of the mares used in this trial were run with a stallion afterwards and are still under observation. The effect of the vaccine on conception may be determined subsequently.

3. Production of PZP immuncontraceptive vaccine

The method for extracting zona pellucida (ZP) was given to us by Dr Janine Duckworth (Landcare Research, Lincoln), who based her protocol on the methods described by Oikawa (1978) and Dunbar & Raynor (1980). Many different types of PZP vaccine have been produced (Table 2) but we choose to develop a vaccine containing 400 gg of PZP protein plus MPL+TDM+CWS adjuvant. This adjuvant was chosen as Freund's adjuvant is expressly forbidden for use in horses by Massey University's Animal Ethics Committee.

Collection of ovaries

Pig ovaries were collected from mixed-age sows at Levin, Hastings, Wanganui and Burnham abattoirs and were frozen (-20°C) until required. Ovaries were thawed at room temperature prior to processing. Approximately 60 ovaries were processed at one time to enable storage of zona pellucida material in batches, as the zonae pellucidae agglutinate if frozen after extraction.

ZP extraction

To keep the extraction procedure clean, buffers and glassware were autoclaved. Equipment that could not be sterilised in this way was washed with 70% ethanol and then sterile PBS.

When thawed, the ovaries were ground up using a hand-driven meat-mincer. Citrate/EDTA buffer was used for washing the ovaries and minced tissue through the mincer and preventing the zona pellucida material from sticking to the mincer. The Citrate/EDTA buffer had a pH of 7.2 and contained 2 mM tri-sodium citrate plus 2 mM di-sodium EDTA.

Ovaries were put through the mincer three times, after which the mincer was flushed with buffer to ensure that all tissue and follicular fluid was collected. This slurry of minced tissue and buffer was mixed for 10 minutes using a magnetic stirrer.

The slurry was then filtered through a series of brass sieves into a glass bowl, and each sieve was thoroughly flushed with buffer. The sieves were 2 mm, 150 Mm, 106 Mm and 75 Mm. Large sieves were used to remove extraneous tissue while the finer sieves separated the oocytes and zona pellucida material. The sieving process was repeated 3 times, after which the filtrate from the 75 Mm sieve was washed on to a watch glass and assessed for zona pellucida material. Numerous ova, zona pellucida and cumulus cells were found in this filtrate. The contents of the glass bowl and other sieves were also checked for zona pellucida. Under a differential interference contrast (DIC) microscope, sub-samples of the filtrate were used to estimate the total number of ZP extracted.

Percoll gradient separation

Zona pellucida may be removed from the filtrate by hand-picking them using a mouth pipette, or by using Percoll (density 1.13g/ml. Sigma, cat. no. P-1644) for a gradient separation. The method for Percoll gradient separation was obtained from Dr Anne Kitchener (Biological Sciences Dept, Newcastle University, Australia - unpublished data).

15 ml of the zona pellucida fraction was carefully layered over a 15ml cushion of 20% Percoll. (A 20% gradient of Percoll is prepared in phosphate buffered saline (PBS), pH 7.2.) This was centrifuged at 600 g for 15 minutes. The zona pellucida material sits in a layer over the Percoll, and other tissue sinks to the bottom. The top layer and part of the Percoll layer were collected using a pasteur pipette, and placed on the 75 m sieve. This was washed with citrate buffer to remove the Percoll, and the resulting ZP-rich aliquots were stored in 20 ml glass vials at -20°C.

Formation of the vaccine

Zonal pellucida-rich aliquots were thawed and spun in a centrifuge at 1000 g for 20 minutes, after which the zona pellucida material formed a pellet at the bottom of the vial. The supernatant was removed and zona were resuspended in a sterile PBS. This procedure was repeated twice to remove citrate/EDTA buffer.

The zona pellucida material from approximately 160 ovaries was then mixed with 2 ml sterile saline which was then mixed with the adjuvant MPL+TDM+CWS (SigmaChemical Co, Germany). This mixture, two doses of the vaccine, was stored at 4°C until use. Before use it was shaken vigorously and was then injected intramuscularly.

Approximately 150 zona were extracted per ovary. A total of four doses of vaccine, each containing 400 Mg of protein were made. According to Dunbar & Raynor (1980), 30 zona pellucida are required to form 1 gg of protein, thus approximately 80 ovaries were used to make each dose of vaccine.

4. Serological responses of thoroughbred mares to PZP vaccine

4.1 VACCINATION OF MARES

Six thoroughbred mares held at the Veterinary Large Animal Teaching Unit at Massey University were used in this study.

They were randomly allocated to one of three treatments:

Control - The two mares were managed as the other treatment animals but were not given a vaccine

Massey vaccine - two mares were injected with the Massey vaccine (400 Mg) on 11 and 28 September 1998. The vaccine was given intramuscularly.

USA vaccine - two mares were injected with the vaccine sourced from the USA on 11 and 28 September 1998.

4.2 BLOOD SAMPLING

Blood samples were taken from all 6 mares on 11 and 28 September and on 15 October 1998. Blood samples were placed on ice and centrifuged immediately (3000 rpm for 10 minutes) and the serum was removed.The serum was divided into 2 aliquots and stored at -20 °C until sent to the USA for analysis.

4.3 SEROLOGICAL RESPONSE TO VACCINE

There was no change in the serum level of zona pellucida IgG levels in the control mares throughout the trial (Table 3). The serological response of one of the mares (#6) given the American vaccine was greater than the mean of the response seen in the Kaimanawa mares (from 0.104 to 2.957). One of the mares given the Massey vaccine (#9) showed a response (from 0.200 to 1.030) similar to that seen in the Kaimanawa mares (Table 3). The results from the second mare given the American vaccine were not received from the analysing laboratory nor were the results from the third blood sample from the second mare which was given the Massey vaccine (Table 3).

4.4 LIMITATIONS OF VACCINE PRODUCTION SYSTEM

There are two major problems with the mass production of zona pellucida immunocontraceptive vaccine in New Zealand.

There are about 40 000 farmed sows in New Zealand and about 15 000 of these are killed each year, that is about 300 a week. The number of sows killed at individual abbatoirs are small, with many abbatoirs killing less than 40 sows per week. These sows come through at different times, and the collection of sufficient ovaries to produce any amount of vaccines is logistically difficult and expensive. About 80 ovaries from adult sows are needed to produce one dose of vaccine using the methods described above. This is more than the weekly sow kill from most abbatoirs.

We asked meat inspectors from the Ministry of Agriculture and Fisheries to collect ovaries for us and they did so as a short-term favour. In the long-term, these inspectors would have to be renumerated for collecting the ovaries, as it adds to their work load.

The method used is a time-consuming and crude practice. A technique for collecting the zona pellucida more effectively would have to be developed. There may be specialist equipment available for this, but none is described in the literature.

4.5 DISCUSSION

The vaccine produced at Massey University was effective in causing a serological response in a thoroughbred mare. Thus it is possible to produce an immunocontraceptive vaccine from porcine zona pellucida using established techniques and quite limited equipment and chemicals. Mass production is difficult, however, for the reasons mentioned above.

5. Conclusions

The PZP immunocontraceptive vaccine sourced from the University of Georgia produced a definite serological response in captive Kaimanawa mares. This vaccine and the vaccine produced at Massey University also produced a definite serological response in thoroughbred mares.

The mass production of such a vaccine is very difficult in New Zealand because of the difficulty of harvesting sufficient numbers of sow ovaries. The methods involved in producing the vaccine are crude but effective.

The vaccine itself is a suspension which readily settles out making it difficult to use with confidence in a dart under field conditions.

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Table 1. The mean (standard error) serum anti-zona pellucida IgG response of captive Kaimanawa mares to a porcine zona pellucida based immunocontraceptive vaccine (units of absorbance at dilution of 1- 500).

Date vaccine administered	Control group	Vaccine X 2	Vaccine X 3
30 Jul 98	0.120 (0.0149)	0.148 (0.0065)*	0.138 (0.0115)*
13 Aug 98	0.173 (0.0266)	0.186 (0.0319)*	0.139 (0.119)*
27 Aug 98	0.150 (0.0481)	1.071 (0.4350)	0.417 (0.816)*
17 Oct 98	0.119 (0.0074)	1.123 (0.32335)	0.571 (0.09730)

Table 2. Types of PZP immunocontraceptive vaccine.

Composition of innoculation	Adjuvant	Protein equivalent	Authors
0.5 ml, pzp in phosphate buffer + 0.5 mL adjuvant	Freund=s complete adjuvant (FCA)	5000 zona pellucidas = 64.3 Mg zp protein	
as above	Freund's incomplete adjuvant (FIA)	as above	Kirkpatrick et al., 1990
aqueous pzp + adjuvant as above	FCA FIA		Turner et al., 1997
pzp + adjuvant as above	synthetic trehalose dicorynomycolate glycolipid (25 mg/ml) + squalene oil as above	400 Mg zp protein 200 Mg zp protein	Willis et al., 1994
zp + adjuvant as above	As above	2000 zona pellucidas = 66 Mg zp protein 1000 zona pellucidas = 33 Mg zp protein	Dunbar & Raynor, 1980
(3 parts zp, 1 part adjuvant) 5000 zp 2000 zp	Amphogel	2000 zona pellucidas = 27.4 Mg zp protein 5000 zona pellucidas = 68.5 Mg zp protein 20 000 zona pellucidas = 274 Mg zp protein	Liu et al., 1989
400ug + adjuvant	RIBI		Fayrer-Hosken (pers. comm)

Table 3. The changes in serum anti-zona pellucida IgG levels (units of absorbance at dilution of 1 - 500) in thoroughbred mares to a locally manufactured immunocontraceptive vaccine based on porcine zona pellucida protein and one imported from the USA.

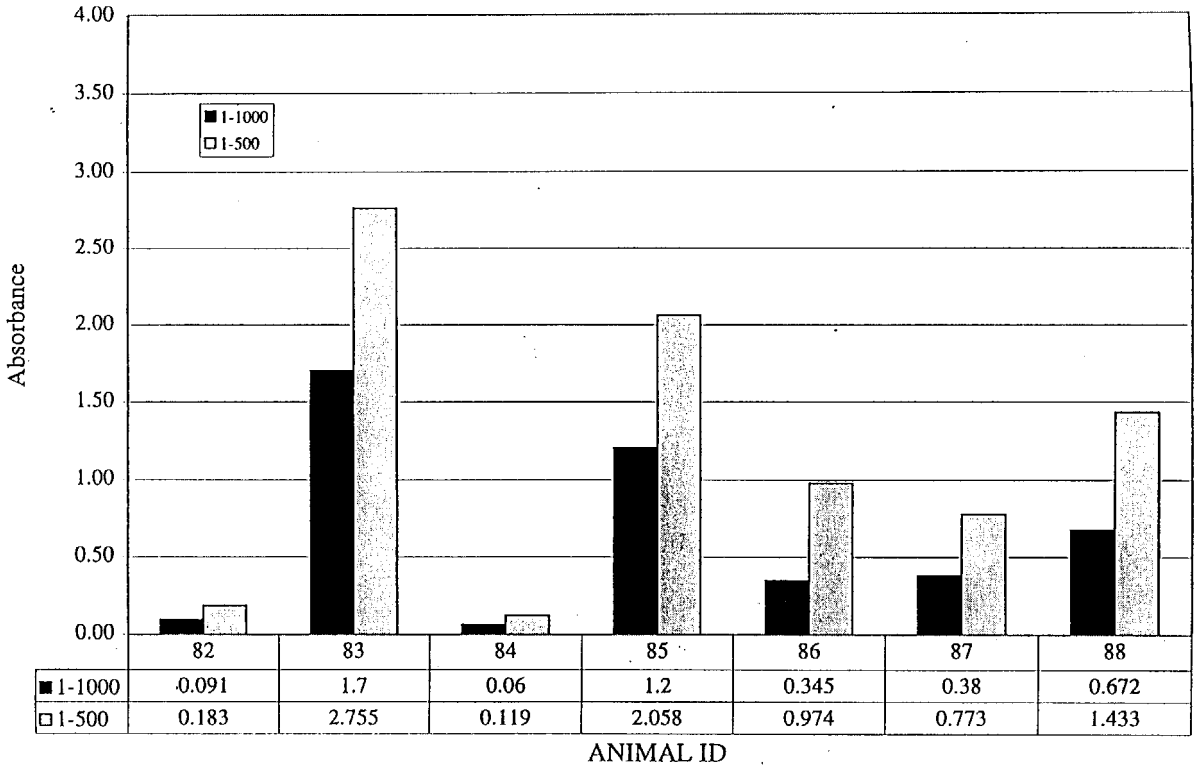
Treatment Animal	Control #92	Control #19	VaceL #29	VaccL #9	VaccA #90	VaccA #6
11 Sep 98	0.173	0.121	0.147	0.204	Na	0.139
28 Sep 98	0.246	0.149	0.153	0.367	Na	Na
15 Oct 98	0.213	0.104	Na	1.433	Na	3.746

Control - mares were blood sampled but did not receive a vaccine

VaccL - mares were vaccinated with a vaccine produced at Massey University

VaccA - mares were vaccinated with a vaccine obtained from the USA

Na - the results from these samples were not received from the laboratory in Georgia, USA.



	82	83	84	85	86	87	88
1-1000	0.091	1.7	0.06	1.2	0.345	0.38	0.672
1-500	0.183	2.755	0.119	2.058	0.974	0.773	1.433

Figure 1. The individual serological response (serum anti-zona pellucida IgG levels) of captive Kaimanawa mares on 17 Oct 1998 after receiving two porcine zona pellucida based immunocontraceptive vaccine delivered by intramuscular injection on 30 Jul 1998 and 13 Aug 1998.