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Reversibility of action and safety during pregnancy of immunization against porcine zona pellucida in wild mares (*Equus caballus*)

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Contraceptive management of publicly valued wildlife species requires safeguards to ensure that these populations are preserved in a healthy state. In addition, reversibility of contraceptive effects and safety in pregnant animals are major concerns. A population of wild horses has been immunized against porcine zona pellucida (PZP) over a 12 year period on Assateague Island National Seashore, MD (ASIS). Mares initially received one or two 65 μg inoculations and once a year 65 μg booster inoculations, all delivered by dart. All young mares aged > 2 years were treated with PZP for 3 consecutive years regardless of whether they have bred successfully and they were then removed from treatment until they had foaled. All mares vaccinated for 1 or 2 consecutive years became fertile again and 69% of mares treated for 3 consecutive years returned to fertility. All five mares treated for 4 or 5 consecutive years have also returned to fertility, but over longer periods of time. Mares treated for 7 consecutive years have not returned to fertility, but several, while still infertile, have started ovulating again. There was no difference in survival rates between foals born to treated and untreated mares, and PZP treatment of pregnant mares did not affect subsequent fertility of their female offspring.

Introduction

Contraception is used increasingly to manage valued wildlife species; however, concern by the public for these animals requires safeguards to ensure that these populations will be preserved in a healthy state and with the potential to propagate successfully in the future. This is particularly true for species such as the North American wild horse, genetically valuable and rare captive exotic species in zoos, and animals with highly developed social behaviours, such as elephants.

In the USA, all wild and feral equids inhabiting public lands are protected under federal law, and public scrutiny of management by government agencies is intense. Where fertility control is used as a management technique, reversibility of contraceptive effects is crucial to the intelligent management of wild horses and is perhaps the single largest public issue. The only contraceptive agent used to treat wild horses routinely in the USA to date has been

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porcine zona pellucida (PZP). Most PZP studies to date have focused on contraceptive efficacy (Kirkpatrick *et al.*, 1990a, 1995a,b, 1996a,b; McShea *et al.*, 1997; Turner *et al.*, 1992, 1997) and short-term safety (Kirkpatrick *et al.*, 1991a, 1992, 1995a; Turner *et al.*, 1996), but certain management applications require a more complete understanding of long-term effects and safety in pregnant mares. In certain species, such as white-tailed deer, or captive exotic species in poor health, or older animals that have already made genetic contributions, reversibility of contraceptive effects is not an issue and often the goal is to prevent animals becoming pregnant in their declining years. However, in other cases, the management goal may be simply to delay reproduction until a later time to keep populations under control, or to allow for later breeding of genetically valuable animals to maintain genetic diversity in the population. In these latter cases, reversibility of contraceptive effects becomes a major issue.

The question of vaccine use in pregnant animals, the possible health effects on pregnancies in progress and the health and subsequent fertility of animals whose mothers were treated while they were *in utero* must also be considered. Again, in some species, for example white-tailed deer, in which seasonal breeding occurs and gestation is approximately 6 months, this is not a significant issue and these animals may be treated at any time during the other 6 months without danger of treatment during pregnancy. However, it is unlikely that large numbers of wild horses (gestation = 340 days) or elephants (gestation = 22 months) can be treated without including pregnant animals.

The published literature regarding PZP provides a diverse and often conflicting view of the reversibility of this vaccine. Reversibility has been documented after a single year of treatment in domestic horses (Liu *et al.*, 1989), wild horses (Kirkpatrick *et al.*, 1991a), white-tailed deer (Turner *et al.*, 1996) and African elephants (Fayrer-Hosken *et al.*, 2000). However, in other species, including dogs (Mahi-Brown *et al.*, 1985; Brandon *et al.*, 1998), rabbits (Wood *et al.*, 1981; Skinner *et al.*, 1984) and baboons (Dunbar *et al.*, 1989), PZP treatment indicates long-term impairment or complete cessation of ovarian function. Although no data have been presented to indicate deleterious effects on fetuses when the mother has been vaccinated during the pregnancy, this issue is nevertheless of concern with regard to the safety of the vaccine.

A herd of wild horses has inhabited Assateague Island National Seashore, MD (ASIS), for more than 300 years. The national seashore is charged with maintaining a herd of these horses as part of the island's historic and cultural resource, but the delicate ecology of the barrier island requires some form of population management. At the same time, there is concern about loss of genetic diversity through population control programmes, which remove horses and, therefore, genes, from the island forever. ASIS implemented immunocontraceptive research in 1988 and a fertility control management programme in 1994, based on the application of PZP to this population. In the present paper, the following are reported: (i) reversibility of contraception in treated mares over a 12 year period; (ii) survival of foals born to treated mares over the period 1988–1999; (iii) maintenance of pregnancy and live births among 26 mares treated while pregnant; and (iv) fertility of mares that were *in utero* at the time of PZP treatment of their mothers. On the basis of the accepted mechanism of the PZP contraceptive vaccine, the first hypothesis was that contraceptive effects would disappear in most animals, and the second hypothesis was that the vaccine would not interfere with pregnancy or the survival or fertility of the foals.

Materials and Methods

Study animals

The entire ASIS wild horse herd is observed for band composition and counted monthly. All individual animals are known by unique markings and band affiliations. Reproductive and

Table 1. Contraceptive reversibility in Assateague Island mares treated annually for 1–7 consecutive years with porcine zona pellucida (PZP) vaccine

Consecutive years of treatment	*n*	Interval between last treatment and pregnancy (years)[a]							
		1	2	3	4	5	6	7	8
1	16	14 (88)	14 (88)	16 (100)	–	–	–	–	–
2	5	5 (100)	–	–	–	–	–	–	–
3	32	5 (15)	12 (39)	21 (66)	22 (69)	–	–	–	–
4	3	0 (0)	0 (0)	1 (33)	2 (66)	0 (0)	0 (0)	0 (0)	3 (100)
5	2	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	–	–
7	5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a]Number (%) of mares returning to fertility. Number and percentage are cumulative for each group.

genealogy data for each individual animal have been maintained since 1974. The population has ranged in size from 43 horses in 1974 to 172 in 2000.

Vaccination

PZP glycoprotein was isolated and extracted by a modification of the method described by Dunbar *et al.* (1980) and stored at –44°C until used. The initial inoculation consisted of 65 µg PZP emulsified in Freund's complete adjuvant (FCA) and all subsequent inoculations were given with Freund's incomplete adjuvant (FIA) as described by Kirkpatrick *et al.* (1990a). All inoculations were administered i.m. remotely, using a 1.0 cc Pneu-Dart® dart (Pneu-Dart, Inc., Williamsport, PA). A single 65 µg PZP booster inoculation was given annually to those mares in which contraception was desired for > 1 year (Kirkpatrick *et al.*, 1991a). Initial inoculations were always administered in March, whereas booster inoculations were administered in either March or August–September. Contraception was considered successful if no foal was born during the 12 months after treatment. Depending on their role in the original study or their place in the management plan, mares were treated with the PZP vaccine for 1, 2, 3, 4, 5 or 7 consecutive years.

Remote pregnancy testing

Remote pregnancy testing was performed on 97 mares between 1988 and 2000, by measuring urinary or faecal oestrone conjugates (E1C) concentrations, or both, as described by Lasley and Kirkpatrick (1991) and Kirkpatrick *et al.* (1990b, 1991b), and immunoreactive pregnanediol-3-glucuronide-like progesterone metabolite (iPdG) concentrations, as described by Kirkpatrick *et al.* (1990c). Urine or faeces were collected directly from the ground following an observed excretion from an individual mare, placed on ice packs for approximately 12 h and frozen until assayed. Collection of these faecal samples was conducted from late August to December, or during the first third of pregnancy in horses.

Foal survival

Foals were defined as surviving if they lived for 12 months after birth.

Results

Of 53 mares treated with PZP for 1, 2 or 3 consecutive years, the reversibility of contraceptive action and a return to fertility (pregnancy) was 100, 100 and 68.8%, respectively, over a 4 year

period after the last PZP treatment (Table 1). Only a limited number of mares ($n = 10$) were treated for > 3 consecutive years, but 100% of the mares treated for 4 or 5 consecutive years returned to fertility, whereas none of the mares treated for 7 consecutive years has yet returned to fertility over a 7 year interval since the last PZP treatment.

Of 246 births between 1976 and 2000 among mares not treated with the PZP vaccine, 206 foals (83.7%) survived to 1 year of age. In a study of a subset of these 246 births, 76 of 86 (88.4%) foals born to mares never exposed to the PZP vaccine survived to 1 year (Keiper and Houpt, 1984). Of 80 foals born to mares that were treated with PZP during the course of their pregnancy between 1988 and 2000, 67 (83.8%) survived to 1 year. Of these 80 pregnancies among the PZP-treated mares, 26 were first diagnosed pregnant between month 8 and month 11 before parturition and all 26 mares produced live foals.

Fourteen filly foals that were born to mares exposed to the PZP vaccine during the course of their pregnancies have now lived to ≥ 6 years of age. Of these 14 foals, eight were permitted to breed without PZP contraception upon reaching sexual maturity at 3 years old, and all eight have produced live foals (11 in total) that have survived to at least 1 year of age.

Discussion

Safety must be a major component of any wildlife contraceptive programme because of its potential application to genetically valuable and often publicly valued species. Reversibility of contraceptive action is one of the primary concerns, at least for certain species such as wild horses, free-ranging elephants and captive exotic species. The mechanisms of action of the PZP vaccine are not entirely understood, but there are several reliable models to explain the basic principles underlying its ability to inhibit fertility (Shivers and Liu, 1982; Florman and Wassarman, 1985). Administration of the vaccine to mammals results in a classic humoral immune response and B-cell-mediated production of antibodies against the glycoprotein components of the native PZP, namely ZP3, ZP1 and ZP2 (Skinner *et al.*, 1989). These IgG class antibodies will bind to the zona pellucida of mammalian ova and presumably block fertilization by causing steric hindrance of the zona sperm receptor (Florman and Wassarman, 1985). This model is consistent with the contraceptive effects of the PZP vaccine in wild mares, at least after 1–3 consecutive years of treatment. Although it was not possible to collect blood from the ASIS mares for measurement of antibody titres, similar studies with domestic mares (Liu *et al.*, 1989), deer (Turner *et al.*, 1996; McShea *et al.*, 1997; Miller *et al.*, 2000) and elk (Garrott *et al.*, 1998) support the model. As anti-PZP antibody titres increase, contraceptive effectiveness increases and as titres decrease, fertility recurs. This would explain the rather high degree of fertility recovery and the relatively short period of time necessary for return to fertility of the mares treated for 1–3 consecutive years.

The increased time for return to fertility in animals treated for 4 or 5 consecutive years, and the complete lack of reversibility in the five mares treated for 7 consecutive years, may be the result of sustained high antibody titres from longer periods of treatment. Alternatively, there may be additional mechanisms at work.

Immune responses can generally be partitioned into two general types, one of which is the B-cell-mediated humoral response, which, in the case of PZP, results in a fertilization block without accompanying oophoritis (Afzalpurkar and Gupta, 1997). The second response is a T-cell-mediated response, in which a variety of defensive phagocytic cells descend upon the target tissue and, in the case of PZP, cause moderate to severe oophoritis (Tung *et al.*, 1996). In most mammals, a particular immune response is dominated by one of these major forms of immunity, but not usually to the complete exclusion of the other. Oophoritis may become a serious long-term impediment to ovarian function and, therefore, fertility, usually as a result of

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autoimmune responses and autoantibodies directed against the zona pellucida, or it may be a temporary condition with full recovery at some point (Lou *et al.*, 1996). Thus, the important question with respect to the mares treated for 7 consecutive years is whether the prolonged contraceptive effects are merely a function of sustained high antibody titres or are perhaps a result of a cellular response that has caused physical damage to the ovary.

Previous studies of the long-term effects of the PZP vaccine (Kirkpatrick *et al.*, 1992, 1995a) have indicated decreased oestrogen secretion, but fertility has always recovered except in animals treated for 7 consecutive years. This finding indicates both a humoral response, which interferes with fertilization and perhaps also a cellular response, which causes the mild reduction in oestrogen production. It is noteworthy that immediately after 7 consecutive years of PZP treatment none of the five mares was ovulating. However, a more recent investigation (D. Powell, personal communication) measuring urinary progesterone metabolites has shown evidence of high luteal phase progesterone concentration. Complete elucidation of the mechanisms at work in the mares treated for 7 years remains to be discovered.

Finally, the equal survival among foals whose mothers had and had not been treated during pregnancy strengthens the model for a simple fertilization block as the predominant immunological mechanism in horses. Furthermore, the normal fertility displayed by mares that were *in utero* at the time of their mothers' vaccinations provides further evidence of the safety of this vaccine in pregnant animals and indicates that the antibodies either do not cross the placenta or, alternatively, that their passage represents passive immunization, which would not be expected to last for the 3 years taken for the mares *in utero* at the time of vaccination to reach sexual maturity.

In conclusion, it is clear that, in horses, the contraceptive effects of PZP treatment administered annually for up to 5 consecutive years are reversible, although the time for reversal is highly variable and may extend to 6 or 7 years. The treatment of pregnant animals does not cause harm to the pregnancy, the survival of the foals or the subsequent fertility of the foals. This finding, coupled with the lack of behavioural side effects (Powell, 1999) indicates that the PZP vaccine represents an extremely safe approach to fertility control in wild horses.

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