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Immunocontraception of Captive Exotic Species. I. Przewalski's Horses (*Equus przewalskii*) and Banteng (*Bos javanicus*)

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A contraceptive vaccine made of porcine zonae pellucidae (PZP) was tested in three Przewalski's mares and five banteng cows. The vaccine antigen consisted of the complete family of glycoproteins of the porcine zona pellucida, including the sperm receptor ZP3. All mares and three of five banteng were inoculated with 2 or 3 i.m. injections of approximately 65 μg of antigen given over a 6 week period. Two other banteng received inoculations of only 35 μg of antigen on the same schedule. Two of the three mares and three of five banteng cows were pregnant at the time of inoculation. No new pregnancies, as a result of postinoculation breedings, occurred among either the mares 36 months after 65 μg antigen inoculations or among the banteng for 24 months after 65 μg inoculations. One postinoculation pregnancy resulted among the two banteng receiving only 35 μg of antigen. Differences in fertility between treated and control mares and between preinoculation and postinoculation reproductive performance of the banteng were significant ($P < 0.05$). Urinary ovarian steroid metabolites and behavioral observations indicated follicular development and ovulations were occurring among treated mares during the year following PZP inoculations. PZP immunization produced progressively elevated anti-PZP antibodies in both species, which provided contraceptive protection. PZP immunization appears to be an effective form of contraception in both species. © 1995 Wiley-Liss, Inc.

Key words: contraception, immunocontraception, surplus animals, ungulates

INTRODUCTION

For almost 20 years reproduction in certain species of captive exotic animals has been regulated by means of contraception [Kirkpatrick and Turner, 1985, 1991; Seal,

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1991]. There are several rationales for fertility control in captive populations. First, the problem of surplus captive animals and acceptable management of these animals has become a serious issue for zoos [Lacy, 1991]. Second, fertility inhibition may be indicated for specific animals for reasons of poor health, undesirable genetic traits, age, or behavioral problems.

The use of contraception for management purposes has, in a large sense, been neglected in ungulates. A recent survey of birth control in North American zoos [Porton et al., 1990] indicates that almost all efforts at chemical fertility control in zoos have been focused upon carnivores and primates. At the same time, exotic captive ungulates breed with relatively high efficiency, require large spaces for exhibits, and are a group of species most often implicated in controversial management practices [Williams, 1992].

A relatively new strategy for population control of captive exotic ungulates is immunocontraception. The principle of immunocontraception involves stimulating the target animal's immune system to interfere with some critical reproductive event. Upon injection of the respective antigen into the target animal, antibodies are produced against some molecule requisite to successful reproduction. One such strategy is to inhibit the protein receptor for sperm attachment on the ovum. The mammalian egg is surrounded by a noncellular membrane known as the zona pellucida (ZP). The ZP consists of several glycoproteins, at least two of which, ZP3 [Florman and Wassarman, 1985] and ZP4 [Hasagawa et al., 1992], have been postulated to be implicated in sperm recognition and attachment. The glycoprotein composition of ZP3 has been identified in a number of species, and its function as a sperm receptor is presumed across these species as well. The large number and diversity of species, now numbering over 30, which can be successfully contracepted with porcine ZP support a common physiological action. Heteroimmunization of a mammal with an entire ZP preparation or purified ZP3 raises antibodies which bind to the sperm receptor and cause steric hindrance, which in turn prevent recognition and binding by sperm [Liu et al., 1989]. Porcine ZP (PZP) has been an effective inhibitor of fertilization in a wide variety of nonhuman primates [Paterson and Aitken, 1990], rabbits [Wood et al., 1981], dogs [Mahi-Brown et al., 1985], humans [Sacco, 1977], horses [Liu et al., 1989; Kirkpatrick et al., 1990a, 1991, 1992a, in press], white-tailed deer [Turner et al., 1992], and a number of captive exotic species [Kirkpatrick et al., 1992b, 1993]. The ability of the PZP antigen to raise antibodies which block fertilization across such a remarkable breadth of species suggests the mammalian ZP3 receptor is highly conserved and underscores the antigen's potential usefulness as a fertility inhibitor in captive exotic species.

Based on previous research, the contraceptive effectiveness of the PZP vaccine can be presumed in a broad spectrum of mammalian species, but actual contraceptive efficacy must be demonstrated on a species-by-species basis. Thus, this pilot study was the first attempt at testing the PZP vaccine in captive zoo species and was carried out to test the vaccine's effectiveness in reducing fertility in a small number of Przewalski's horses (*Equus przewalskii*) and banteng (*Bos javinacus javinacus*). In addition to examining contraceptive effectiveness, anti-PZP antibody titers were measured in both species. Urinary metabolites of ovarian hormones were measured in the Przewalski's horses to determine if ovarian estrogen production was within normal ranges and if postinoculation ovulations were occurring.

MATERIALS AND METHODS

Preparation of the PZP Vaccine

The antigen was prepared as described by Gwatkin et al. [1980] and Dunbar et al. [1980]. Briefly, frozen/thawed porcine ovaries were minced in cold phosphate buffered saline (PBS) using a ganged razor-blade apparatus. The oocytes were separated from other tissues, including granulosa cells, by screen filtration, counted, and homogenized in a Potter-Elvehjen homogenizer. The zonae were isolated on a 48 μm screen, heat solubilized at 70°C, and stored frozen in PBS.

Study Animals

Seven Przewalski's mares and five banteng cows at the Zoologischer Garten Koln were selected for the study. Two of the three PZP-treated mares (Noes, age 12; Aida, age 20) were of proven fertility (multiparous) and one (Alina, age 2) was of unproven fertility. Noes was selected for contraception because she harbors the "fox allele," a trait causing undesirable coat color. Aida was selected because of advanced age and a chronic respiratory disease. Alina was selected because the zoo did not want to breed her at age 2. One primiparous (Natasha, age 3) and three multiparous mares (Ariane, age 4; Anushka, age 7; Lady, age 9) served as untreated controls. Previous results with feral mares receiving only saline and adjuvant [Kirkpatrick, 1990a] have already indicated that adjuvant alone does not interfere with fertility; thus the four control animals were not treated in any way.

The five PZP-treated banteng were selected primarily because of a desire to limit the zoo's herd size, but one nulliparous cow (Beli, age 2) was highly inbred. The other four cows (Sabah, age 10; Bali, age 8; Birma, age 4; Siri, age 3) were multiparous. These five PZP-treated banteng cows served as their own controls, and fertility records prior to immunization were compared to fertility records postimmunization. Differences in fertility between treated and untreated Przewalski's horses and between pre- and posttreatment banteng were tested for significance by binomial probability distribution [Freedman et al., 1978]. The horses and banteng were stabled in individual stalls after zoo visiting hours and were fed commercial concentrates and hay in the evening and again each morning.

Monitoring of Ovarian Endocrine Function

Ovarian endocrine function was monitored in the three PZP-treated Przewalski's mares by means of urinary steroid metabolites, as described in domestic mares by Evans et al. [1984], in feral mares by Kirkpatrick et al. [1990a,b, 1991, 1992a], and in Przewalski's mares by Monfort et al. [1991]. On several occasions when behavioral estrus was observed, urine samples were collected by aspirating the urine directly from the ground of enclosures or stalls during the mornings. Urine collection was not possible during all observed periods of estrus because of keeper time constraints. The samples were frozen and stored until the time of assay. Behavioral estrus was determined on the basis of 1) raised tail when stallions attempted mounting, 2) eversion of the clitoris, 3) absence of kicking or biting by the mare during attempted or successful mountings, and 4) copulation, as described by Waring [1983].

Immunoreactive urinary estrone conjugates (E_1C) were measured by enzyme immunoassay as described by Shideler et al. [1990] and validated for the Equidae [Kirkpatrick et al., 1990a,b,1991,1992a, in press]. Immunoreactive pregnanediolglucuronide-like progesterone metabolites (iPdG) were analyzed by enzyme immunoassay as described by Kirkpatrick et al. [1990b] and validated for the Equidae [Kirkpatrick et al., 1992a, in press]. The purposes for monitoring urinary estrogen and progesterone metabolites were threefold. First, urinary E_1C concentrations were examined to determine if they increased at times of observed behavioral estrus, an endocrine event correlated to ovarian follicular maturation. Second, urinary E_1C concentrations were examined to determine if they fell within normal ranges previously reported for domestic [Daels et al., 1991] and Przewalski's mares [Monfort et al., 1991]. This also would confirm or reject the occurrence of normal ovarian endocrine function, at least with respect to estrogen secretion. Finally, sustained elevations (a twofold increase over approximately 20 days) of iPdG, between 100 and 400 ng/mg creatinine, were taken as evidence that ovulation had occurred, as described by Kirkpatrick et al. [1992a,b, in press].

Anti-PZP Antibody Measurements

Blood samples were collected opportunistically to minimize handling-induced stress, and the serum was analyzed for anti-zonae antibodies. Among the Przewalski mares, blood was collected from Noes, Alina, and Aida and among the banteng from Sabah, Birma, and Bali. Zonae antibody analysis was accomplished by ELISA methods described by Voller et al. [1986], with modifications previously reported by Liu et al. [1989]. Fifty microliters of 5 μg/ml zona antigen solution in 0.1 M glycine buffer (pH 9.5) was placed in each well of a flat-bottomed ELISA microplate (Sumilon®, No. MS-3496; E & K Scientific Products, Saratoga, CA) and incubated overnight at 4°C. The plate was washed with PBS-TWEEN and incubated for 1 h each with 50 μl of the following PBS-TWEEN diluted reagents and subsequent washings: horse serum 1:1,000 and banteng serum 1:500; 2) biotinylated goat anti-horse IgG 1:1,000 (Zymed Laboratories, San Francisco, CA) or biotinylated goat anti-bovine IgG 1:1,000 (Kirkegaard and Perry, Gaithersburg, MD); 3) alkaline phosphate avidin 1:1,500 (Zymed) for horses and banteng. Optimal dilutions of reagents for best distinction between positive and negative reactions were selected after checkerboard titrations. Finally, 50 μl substrate solution of 1 mg *p*-nitrophenyl phosphate/ml (5 ml tablets; Sigma Chemical Co., St. Louis, MO) in 10% diethanolamine buffer (pH 9.8) was distributed to each well. The plate was screened for absorbance at 405 nm using an MR 580 Microelisa Auto Reader (Dynatech Laboratories, Alexandria, VA) when the absorbance of the positive reference serum had reached a level close to 0.8 after incubation of approximately 10 min.

The experimental sera were tested in duplicate, and their results were expressed as percentage of the positive reference serum (mean EA_{405}/mean RA_{405}). The reference serum consisted of a pool of sera that had demonstrated anti-zona antibody titers of medium-positive range. Pooled preimmunization sera served as negative controls.

RESULTS

Przewalski's Horses

Prior to PZP inoculations, Noes produced six foals, with an average interfoal interval of 15 months. Noes was approximately 4.5 months pregnant at the time of her first PZP inoculation and delivered a healthy foal 7 months later. Aida produced nine foals prior to PZP inoculations, with an average interfoal interval of 18.7 months. She

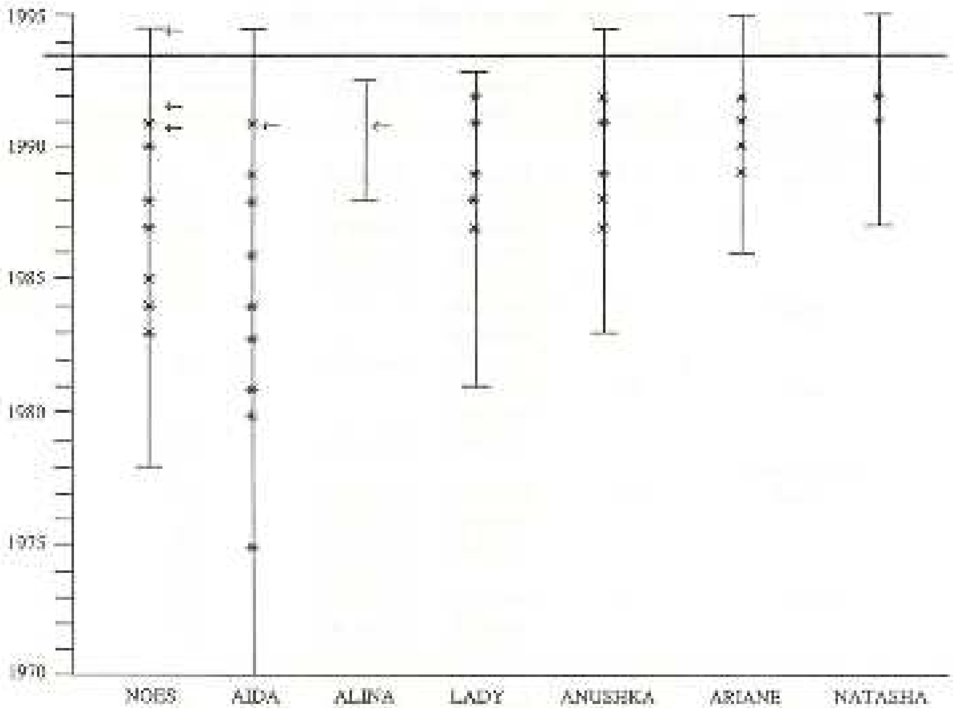

Fig. 1. Pre- and post-PZP inoculation reproductive records for three treated (Noes, Aida, and Alina) Przewalski's mares and untreated control mares. Asterisks represents foals born and arrows the approximate time of PZP inoculations. The line through the top of the figure represents the conclusion of the experiment, when mares were separated from the stallion.

was approximately 1 month pregnant at the time of her initial inoculation and aborted a 4-month-old fetus. None of the three PZP-treated mares produced any foals during the 36 months following inoculations as a result of postinoculation breedings (as opposed to breedings that occurred prior to inoculations). The four untreated control mares all became pregnant during the same period of time and collectively produced eight foals. On the basis of binomial probability distribution, the difference in fertility between PZP-treated and untreated mares was highly significant ($P < 0.001$). Figure 1 indicates the pre- and postinoculation foaling dates for treated and untreated mares.

Inoculation dates and anti-PZP antibody concentrations for the three PZP-treated mares are presented in Table 1. The only mare for which a preinoculation titer was available is Noes, and the value of 4% of the positive reference value is the same as in untreated domestic mares [Liu et al., 1989]. The posttreatment antibody titers were similar in concentration and timing to those found in domestic mares after PZP inoculations [Liu et al., 1989]. In domestic mares, previous research has indicated that antibody titers which are >64% of positive reference standards provide contraceptive protection in mares [Liu et al., 1989]. All treated mares in this study exceeded that titer.

Noes demonstrated behavioral estrus on December 26, 1991, approximately 16 months after her last previous booster inoculation. The timing of this estrus coincided with a dramatic elevation of urinary E_1C, which reached a peak of 840 ng/mg Cr on

TABLE 1. Ages, inoculation dates and antibody titers for all PZP-treated animals

Study animals	Age (years)[a]	Inoculation dates	Bleeding dates	Antibody titers (% positive reference)
E. przewalskii				
Noes	12	03/05/90	03/05/90	4
		03/19/90	03/19/90	35
		04/02/90	04/20/90	112
		09/24/90	06/25/91	105
		08/21/91	08/28/91	95
Alina	2	09/11/90		
		09/24/90		
		10/22/90	04/01/92	108
Aida	20	09/11/90		
		09/24/90		
		10/22/90	05/06/91	85
B. javanicus				
Sabah	10	09/17/90	09/17/90	10
		10/01/90	10/01/90	62
		10/25/90	10/25/90	78
			09/13/91	85
Bali	8	09/17/90	09/17/90	12
		10/01/90	10/01/90	55
		10/25/90	10/25/90	81
			09/13/91	94
Binna	4	09/17/90	10/01/90	63
		10/01/90	10/25/90	80
		10/25/90	09/13/91	88
Siri	4	02/04/92		
		02/18/92		
		03/24/92		
Beli	3	02/04/92		
		02/18/92		
		03/24/92		

[a]Age at beginning of treatment.

December 19, or about 484 days after her last booster inoculation. There was also a rise in urinary iPdG following the E_1C peak, which is coincidental with ovulation (see Fig. 2).

Aida displayed behavioral estrus for 3 days on October 20, 21, 22, 1990, and was bred by the stallion. Elevated urinary E_1C (420 ng/mg Cr) and elevated iPdG concentrations (500 ng/mg Cr) followed the mating, signalling ovulation, 41 days following her initial inoculation (see Fig. 3). This mare again displayed estrus on February 10, 1991, 152 days after her initial inoculation and 3 months following her last previous PZP booster inoculation. Urine samples were not available at this time. She again displayed behavioral estrus and was mated 63–66 days later, on April 14–17, 1991 (or 227 days postinitial inoculation). There was a urinary E_1C elevation coincidental with this estrus, but urinary iPdG did not rise dramatically (see Fig. 4). Aida displayed behavioral estrus and was again mated on May 3, 1991, but urine samples were not available.

The third treated mare, Alina, displayed behavioral estrus on March 15, 1992,

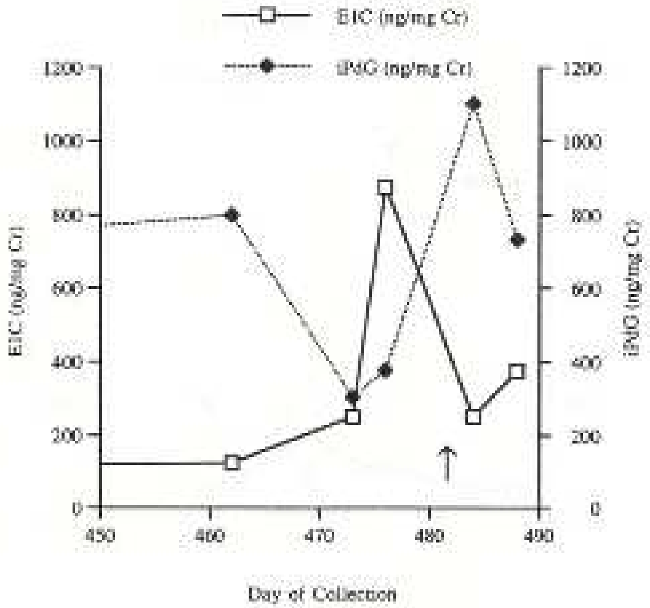

Fig. 2. Urinary E_1C and iPdG concentrations for the mare Noes, 462–488 days following the initial PZP inoculation. A preovulatory E_1C peak is coincidental with an iPdG nadir and is followed by an observed breeding and a luteal phase iPdG elevation, signalling ovulation. Arrow indicates the time of observed behavioral estrus.

19 months following her last PZP inoculation. Urine samples were not collected at that time. In August, 1991, 11 months after her last inoculations, this mare constantly followed the stallion and disturbed him during his interactions with other mares. At the end of August the stallion started to attack Alina and Noes by biting and kicking them. Noes, who could not defend herself with kicks because of ataxia, had to be kept separated. Alina was separated from the rest of the herd by the stallion's chasing her into a distant part of the enclosure. No attacks occurred as long as she stayed there. She often approached the stallion during his visits in that part of the enclosure, sniffed at him, and briefly tolerated his olfactory investigation before kicking at him.

Noes and Alina each developed a small abscess after their third inoculations, and Noes developed a new abscess after each subsequent inoculation. Additionally, Alina displayed lameness in the injected limb for 3 days. The healing process was rapid, and abscesses disappeared within 6 days.

Banteng

Of the three cows receiving inoculations of 65 μg of PZP (Sabah, Bali, and Birma) none produced a calf from postinoculation breedings during the following 32 months. Sabah was approximately 1 month pregnant at the time of her first inoculation and produced a healthy calf 8 months later. During the pretreatment years 1985–1990, Sabah produced five calves, with a mean intercalf interval of 11.3 months. Bali, born in 1982, produced two calves (1988 and 1989), and Birma, born

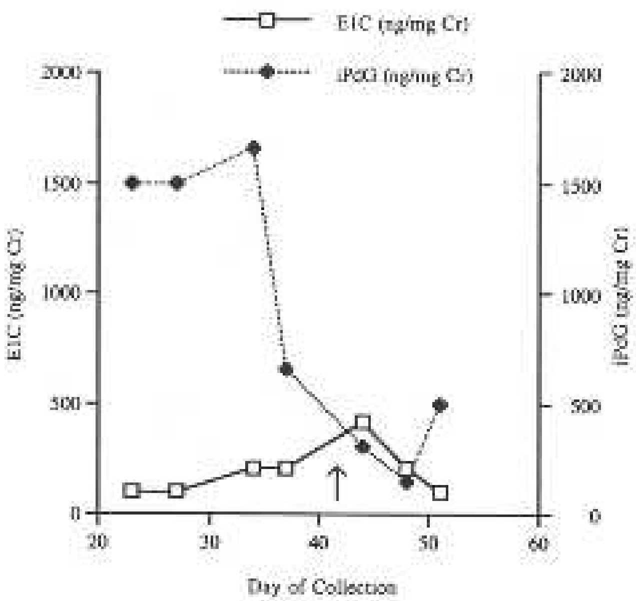

Fig. 3. Urinary E_1C and iPdG concentrations for the mare Aida 23–51 days following the initial PZP inoculation. An E_1C peak is coincidental with an iPdG nadir and a witnessed breeding. Arrow indicates the time of observed behavioral estrus.

in 1986, produced two calves, in 1988 and 1989. Collectively, these three PZP-treated cows produced ten calves during the pretreatment years 1985–1990, which was significantly different from the zero calves born after treatment ($P < 0.01$) (see Fig. 5).

Of the two cows receiving only 35 µg of PZP, one (Siri) produced a calf from a posttreatment breeding, 16 months after her last inoculation. Beli, who was acquired from another zoo and introduced into the herd immediately after her last inoculation in March, 1992, gave birth to a premature calf approximately 4 months later. The calf was estimated to be near term. Siri was also pregnant at the time of her inoculations in March, 1992, and produced a healthy calf a day after her last inoculation. The pre- and posttreatment calving dates for all five cows are shown in Figure 5. The anti-PZP antibody titers for the three banteng inoculated in 1990 with 65 µg of PZP are given in Table 1. There was a progressive elevation of antibody titers after each of the three inoculations, which culminated in substantial titers (94%, 85%, 80%) that correlated with contraception.

The resident bull did not indicate any sexual interest (tending, driving, mounting) toward any of the cows treated in 1990 (Sabah, Bali, and Birma) until August, 1992, at which time he was observed driving Birma. Birma came into estrus again in January and February, 1993. Sabah and Bali also aroused interest by the bull in May, 1993, but no matings were observed. Siri, who was treated early in 1992, came into estrus in June and July, 1992, and was bred and was diagnosed pregnant early in 1993. She produced a healthy calf on July 9, 1993. No abscesses were observed among the inoculated banteng.

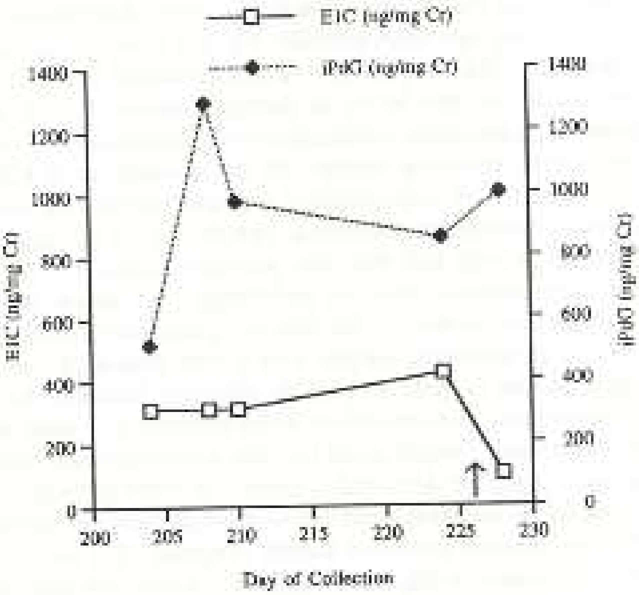

Fig. 4. Urinary E_1C and iPdG concentrations in the mare Aida, 200–230 days postinitial PZP inoculation. Luteal phase iPdG concentrations are elevated at day 205 and begin a gradual decline until Day 227, at which time the nadir coincides with an E_1C peak and a witnessed breeding (indicated by arrow).

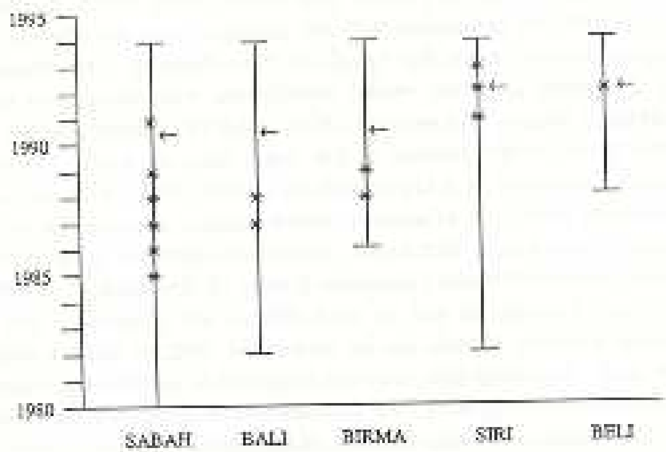

Fig. 5. Pre- and postinoculation reproductive histories for three banteng cows treated with 65 µg of PZP (Sabah, Bali, Birma) and two treated with 35 µg (Siri, Beli). Asterisks indicate calves born, and arrows indicate approximate time of PZP inoculations.

DISCUSSION

These results indicate that multiple PZP immunizations of Przewalski's horse mares and banteng cows with 65 µg of PZP provide contraceptive protection. The contraceptive success with the Przewalski's horses was expected, based on previous success with domestic and feral horses [Liu et al., 1989; Kirkpatrick et al., 1990a, 1991, 1992a, in press], but this study provides the first demonstrated success with a member of the Bovidae. The Przewalski's horses produced anti-PZP antibodies at approximately the same rate and levels as domestic horses [Liu et al., 1989] and exceeded the assumed approximate contraceptive threshold of 64% of positive reference values. In banteng receiving multiple 65 µg inoculations of PZP, substantial antibody titers correlated with contraception. In both species, no conceptions occurred for 36 months following immunization with the full 65 µg dose of PZP, while one of two banteng given only half that dose produced a calf.

Contraceptive effectiveness alone is insufficient to evaluate the usefulness of fertility control agents for captive exotic species, particularly if the contraceptive agent will be used in an individual animal over a long period of time. Ideally, the contraceptive agent should be safe to give to pregnant animals, its contraceptive effects should be reversible, there should be no or minimal long-term health effects, and it should be economically within reach for zoos for routine use. Finally, it would be helpful if the agent could be delivered remotely, in order to reduce stress among treated animals. One of the two mares and two of the three banteng that were pregnant at the time of PZP inoculations produced healthy offspring. The inoculation of pregnant animals was not planned in this study, but it is a reality that must be considered when inoculating large ungulates. The one aborted foal and the premature banteng calf among a total of five treated pregnant animals is unusual, based on previous use of this vaccine. These results are not consistent with the findings of Kirkpatrick et al. [1990a], in which almost 60% of 26 PZP-treated feral mares were pregnant at the time of inoculation yet produced healthy foals. This earlier study with feral mares supports the idea that PZP-induced antibodies will not interfere with pregnancies in progress or have deleterious effects upon the health of the offspring. The abortion by Aida approximately 2 months after her initial inoculation was thought to be caused by fungus-contaminated silage, and several other non-PZP-treated species which were eating the same grain stocks aborted at the same time as Aida. The one abortion among the banteng occurred in a highly inbred animal (Beli) who was also infected with *Tritrichomonas foetus*, a condition which causes abortion in up to 20% of infected animals [Grunert and Berchtold, 1982]. Despite the accumulation of data from other studies with PZP-treated pregnant horses [Kirkpatrick et al., 1990a, 1991, 1992a] that suggests a complete lack of fetal effects, the possibility that PZP immunization can cause abortion should not be dismissed without further study.

The pilot study described here was not designed to address the issues of reversibility of contraceptive effects or of PZP immunization upon ovarian endocrine function. Both are the subjects of other studies [Kirkpatrick, in press]. However, taken together, the urinary endocrine and behavioral data from the study are not without value. First, the urinary E_1C values and patterns for PZP-treated mares were similar to those measured in semi-captive Przewalski's mares by Monfort et al. [1991]. Urinary estrone conjugate elevations consistent with concentrations sufficient for ovulation in this species were noted sporadically over the 2 year period, but, due to infrequency of sample collection, no conclusions on patterns or normality of ovarian cyclicity could be made.

Second, the occurrence of luteal phase urinary iPdG elevations after two of the three periods of behavioral estrus monitored provides indirect evidence for the occurrence of ovulation, and these data are consistent with the findings of Liu et al.

[1989], in which serum progesterone concentrations in PZP-treated domestic mares indicated that ovulation had occurred and that normal luteal function was occurring. In feral horses, a single year of treatment did not interfere with normal reproduction in year 2 of the study [Kirkpatrick et al., 1991], and after 3 consecutive years of treatment the majority (60%) of treated mares were still demonstrating ovulatory estrous cycles [Kirkpatrick et al., 1992a, in press]. The fact that reproductive behaviors were associated with elevated urinary E_1C and an iPdG nadir strengthens the inference that ovarian dysfunction had not occurred during the year following PZP treatment. The last urine collections were carried out in July, 1991, and endocrine data are not available after this date, yet all three PZP-treated mares continued to display estrous behavior and matings after this time. Taken collectively, behavioral observations and sporadic E_1C and iPdG elevations suggest that follicular recruitment and maturation, with subsequent ovulation, likely occurred on occasion, but normal luteal phases and ovarian function could not be assessed.

Differences among species may also be important with regard to PZP effects upon ovarian endocrine function. Although no urinary endocrine data were available for the banteng, keepers witnessed no signs of estrus during the year following inoculations. This suggests that the contraceptive mechanism involved more than a fertilization block. However, all three cows treated in 1990 showed clinical signs of estrus by June, 1992. The return of estrus behavior in treated animals is encouraging and suggests that ovarian function was not permanently depressed after a single year of treatment. Although antibody titers were not available for banteng after September, 1991, previous studies with domestic horses [Liu et al., 1989], feral horses [Kirkpatrick et al., 1991], and white-tailed deer [Turner et al., 1992] indicate that the probability of contraceptive antibody titers 18 months after three inoculations is very small.

These are important considerations because in species such as rabbits [Wood et al., 1981], dogs [Mahi-Brown et al., 1985], and some primates [Dunbar et al., 1989; Paterson et al., 1992], previous studies have demonstrated serious ovarian endocrine dysfunction after PZP treatment. The various ovarian effects described in these studies have included depletion of ovarian follicles with subsequent declines in estrogen secretion and ovulation failure. In some species, such as rabbits and dogs, the effects of even short-term treatment appear to be severe, while in others, such as marmosets [Aitken et al., 1984], bonnet monkeys [Bamezai et al., 1986], squirrel monkeys [Sacco et al., 1987], and horses [Liu et al., 1989], the effects are minor or transient. Long-term treatment of horses however, from 4–7 years, leads to a cessation of ovulation and depressed estrogen concentrations in horses [Kirkpatrick et al., in press]. Additionally, care must be taken when comparing the results from earlier studies with rabbits, dogs, and primates and the two species in this current study because of the significant dose differences. The earlier studies utilized repeated doses of 100–5,000 μg of antigen, while this study utilized only doses of 65 μg or less.

The antagonistic interactions between the stallion and Alina and Noes are probably unrelated to treatment. First, the urinary endocrine data reflect normal estrous cycles, and there is no reason to expect that changes in behavior were related to endocrine status. Second, although quantitative behavioral data were not collected over the entire study period, the behaviors displayed by the stallion towards these two mares strongly resemble that which was described by Mackler and Dolan [1980] and Kolter and Zimmermann [1988] for Przewalski's horses at San Diego and Koln Zoos, respectively. Finally, no similar changes in behavior have been witnessed among PZP-treated feral horses [Kirkpatrick et al., 1990a, 1991, 1992a]. Whether the escalation of aggressiveness of the stallion was caused by disturbed socio-sexual behaviors of the treated mares cannot be determined by this study. More systematic behavioral observations should be conducted during the course of future studies with any form of contraception.

The occurrence of minor abscesses in the Przewalski's mares at the injection site is probably unrelated to the use of Freund's Complete adjuvant. This particular adjuvant has proved effective in eliciting antibody production but is often accompanied by the formation of abscesses. However, Noes and Alina developed abscesses only after their third and each subsequent inoculation, which consisted only of Freund's Incomplete adjuvant. Among 52 different PZP-treated feral horses on Assateague Island National Seashore, only five such abscesses have been observed [Kirkpatrick, unpublished data], and more recently, among 70 captured feral mares in Nevada inoculated twice with PZP, not a single animal produced an abscess. These data from feral horses and the absence of abscesses in the banteng suggest that Przewalski's horses may be more sensitive to the incomplete adjuvant than other species. Additionally, the possibility of sera testing positive for tuberculosis from animals that have been previously inoculated with Freund's Complete adjuvant, and the importance of this issue among zoo animals, indicates that alternative adjuvant protocols should be developed.

Finally, while concern about reversibility of contraceptive action and long-term effects on ovarian function are extremely important in the increasing application of this immunocontraceptive among captive exotic species, these issues and the current paucity of data need not detract from the vaccine's value to zoos in all cases. In the context of this study, it was the hope of the Koln Zoo that two of the three Przewalski's horses would not breed again. The third mare will be permitted to breed in the next year or two; thus, the issue of long-term effects was not relevant in this case. The banteng will also be permitted to breed in the future, and PZP immunocontraception is viewed only as a short-term approach. Until more is known about the long-term effects, recommendations for the use of the vaccine will remain conservative, focusing on short-term use in SSP or EEP animals or long-term use in aged, ill, or genetically undesirable animals.

CONCLUSIONS

1. Porcine zonae pellucidae immunization produces rapidly elevated anti-PZP antibodies in Przewalski's horses and banteng.
2. Substantial anti-PZP antibody titers in excess of 64% of positive reference values provide contraceptive protection in both species.
3. An initial series of three inoculations does not irreversibly interfere with reproductive behaviors in banteng.

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