

IN VIVO BLOCK OF FERTILIZATION IN MARES HETEROIMMUNIZED
WITH PORCINE ZONA PELLUCIDA

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SUMMARY

Groups of 10 feral mares and 6 domestic horses were immunized with porcine zona pellucida (PZP) by 4 monthly injections of 2000-5000 PZP each, and a booster injection of 20000 PZP 6-10 months later. The immune response was reflected in high antibody titers as measured by immunofluorescence with porcine oocytes and subsequent in vivo inhibition of fertility for at least 8 months in most of the mares treated. When antibody concentrations had decreased to lower levels in 4 mares, they conceived normally. There was no clinical evidence of abnormal side effects on ovarian function or on regained fertility following the immunization. In vitro sperm binding studies support that inhibition of binding is antibody-related.

INTRODUCTION

In vivo attempts to reduce fertility in several mammalian species have been reported. Active immunization of female rabbits (Gwatkin, Andersen & Williams, 1980; Wood, Liu & Dunbar, 1981; Skinner, Mills, Kirchick & Dunbar, 1984), bitches (Mahi-Brown, Huang & Yanagimachi, 1982; Shivers, Sieg & Kitchen, 1981) and monkeys (Sacco, Subramanian, Yurewicz, DeMayo & Dukelow, 1983) with porcine zona pellucida (PZP) have resulted in reduced fertility of these species. Similarly, pregnancy was successfully inhibited in mice (Gwatkin, Williams & Carlo, 1977) and rabbits (Gwatkin & Williams, 1978) using hamster and bovine zona pellucida, respectively. Other investigations, utilizing passive immunization, were also successful in inhibiting fertilization *in vivo* in several rodent species when antisera against mouse (Shahani, Padbidri & Rao, 1972; Tilek & Pavlok, 1975), rat and hamster ovaries (Tsunoda & Chang, 1976; Oikawa & Yanagimachi, 1975) were injected into the females.

Natural antibodies directed against zona pellucida have been reported to occur in mares infertile for unexplained causes (Liu & Shivers, 1982). Further attempts to investigate the role of naturally occurring equine antibodies against zona demonstrated immunological crossreactivity of porcine and equine zonae and the *in vitro* reduction of binding of boar sperm to porcine oocytes treated with zona positive equine antisera (Shivers & Liu, 1982).

According to several reports, however, the use of zona components as antigen in active immunization resulted in altered cyclicity and ovarian malfunction. Such side effects were described in the bitch (Mahi-Brown et al., 1982, Mahi-Brown, Yanagimachi, Hoffman & Huang, 1985). In rabbits a decrease in ovarian weight, a marked reduction of follicular development, abnormal follicular differentiation and lack of response to induction of ovulation by human chorionic gonadotrophin were reported (Wood et al., 1981; Skinner et al., 1984). In monkeys fewer ovulated oocytes were recovered following the use of purified porcine zona antigen (Sacco et al., 1983).

The present study was carried out to determine if heteroimmunization of mares with PZP would temporarily inhibit fertilization *in vivo*. If so, is fertility inhibited as a result of ovarian malfunction or is inhibition due to a reduction of sperm binding to oocytes or both?

MATERIALS AND METHODS

Preparation of Antigen

Freshly frozen sow ovaries were obtained from an abattoir. PZP were collected using procedures reported by Gwatkin et al., 1980. The frozen ovaries were thawed in phosphate buffered saline (PBS, pH 7.2) and sliced in a ganged razor blade apparatus (Dunbar B.S., Wardrip N.Y. & Hedrick Y.L., 1980). The resulting suspension was filtered through a series of screens of 210, 150 and 73 μm . The two larger screens trap particles larger than oocytes while the 73 μm screen collects the oocytes on its surface and allows particles smaller than oocytes to filter through.

Oocytes separated as described were counted and then ground by means of a 7 ml Pyrex tissue grinder. The PZP were trapped on the surface of a 48 μ m screen and thus separated from the cytoplasm which was discarded. They were thoroughly rinsed with PBS and 0.5 ml PBS was added per dose of either 2000 or 5000 oocytes. The PZP were heat-solubilized in a waterbath for 30 minutes at 80°C. The screen was then removed and the heat-solubilized PZP aliquoted in single doses.

Immunization adjuvants

One part of Amphogel (aluminum hydroxide gel) was mixed with 3 parts of heat-solubilized PZP for use in the first two inoculations in all the 16 horses of this study. However, in four of the six treated domestic horses it had become apparent that this adjuvant was only moderately effective and therefore it was substituted. Equal parts of zona preparation were emulsified with first complete and then incomplete Freund's adjuvant for the remaining inoculations.

Selection of mares

Two groups of horses were utilized in this investigation:

Group 1: Feral horses

Ten feral mares were captured at a wild horse sanctuary in Northern California. The 5000 acre sanctuary consisted of approximately 200 horses of differing ages. Of these about 120 were mares, 15 were stallions and the remaining horses were neutered males (geldings). Initially, approximately 30 mares were captured and confined to a large area for a period of 6 months without stallions. Prior to immunization, each mare was herded into a restraining chute. Their reproductive tracts were rectally palpated to determine their pregnancy status and whether any abnormalities were present.

Ten mares were selected for this study on the basis of 1) a palpable normal reproductive tract, 2) a non-gravid uterus, and 3) age. The mares selected were estimated to be between the ages of 3 and 10 years. Four of the 10 mares appeared to be maiden mares and the remaining 6 had foals by their sides. Individual mares were identified by their color and markings and the group was marked by roaching their manes. The 10 selected mares remained in a small corral for the period of the initial 4 immunizations. They were then released to their domicile and recaptured 8 months later.

Group 2: Domestic horses

The group of domestic horses was placed on a similar inoculation schedule and received the same doses as the feral mares. These horses provided the opportunity for monitoring of antibody titers developed as a result of the injections and allowed for frequent examination of the reproductive tract for folliculogenesis, ovulation and pregnancy status. The group consisted of 4 presumably fertile mares, one infertile mare with chromosome abnormality (63x0 genotype) and one gelding. The 4 fertile mares were selected on the basis of 1) a palpable normal reproductive tract and speculum examination, 2) age, 3) history of pregnancy, and 4) normal cyclicity. All 4 fertile mares were between the ages of 3 and 8 years, had

a history of never having been serviced by a stallion, and had normal physiological and behavioural estrous cycles. The infertile mare and the gelding served as immunological controls.

Immunization

Serum samples were obtained from all horses (domestic and feral) prior to the initial immunization. A series of 4 intramuscular injections of heat-solubilized PZP was given to each horse at 2-4 week intervals. The initial immunization dose consisted of 5000 PZP, except for 2 domestic horses (the fertile mare ST and the gelding AL) which received 2000 PZP. All the horses received 2000 PZP for the second inoculation and doses were increased to 5000 PZP for the 2 remaining inoculations.

Booster injections of 20 000 PZP were given to the fertile domestic mares when their anti-PZP antibody titers (ZATs) as determined by the indirect immunofluorescence assay, had decreased to low levels. The feral mares were boosted 8 months following their initial series of 4 injections regardless of their ZATs. Serum samples for PZP antibody level determination were obtained prior to each subsequent inoculation from each horse and from the domestic horses additionally every month throughout the 23 month period of this study.

Breeding of mares and test for pregnancy

Eight months following the initial 4 inoculations and being returned to their natural habitat with known fertile stallions, the feral mares were recaptured and rectally palpated for evidence of pregnancy.

The 4 domestic fertile mares immunized were rectally palpated every other day through each estrous cycle for observation of follicular development and ovulation. They were either hand bred or artificially inseminated with known fertile stallions, no more than 24 to 48 hours prior to ovulation. Ultrasonographic scanning 16 to 25 days post ovulation was utilized to detect early stages of pregnancy. Additional pregnancy examinations were performed by rectal palpation at 45 and at 60 days of gestation.

Indirect immunofluorescence assay (IIFA)

The presence of antibodies directed against PZP in the sera of the experimental horses following immunization was determined by IIFA with porcine and equine oocytes.

Procedures used to obtain porcine oocytes were as described above. Prior to the assay, the oocytes were incubated in a small amount of undiluted heat-inactivated fetal calf serum for 1/2 hour at 22°C to render them less adherent. They were then washed three times in PBS by centrifugation in a Fisher microfuge at 50g for one minute and resuspended in PBS at the concentration of 2000 oocytes per ml. Two-fold test serum dilutions were prepared in PBS. One hundred oocytes (= 50 µl) were incubated with 300 µl of each serum dilution for 30 minutes at 22° C and washed 3x in PBS. Following the final centrifugal wash, the oocyte pellet was resuspended in 100 µl of rabbit anti-horse IgG conjugated with fluorescein isothiocyanate (Miles-Yeda Ltd.) diluted 1:10 in PBS, and

incubated for 30 minutes at 22°C in the dark. Following 3 further washings, the oocytes were resuspended in 30 µl of PBS. The treated oocytes were transferred onto slides and the PZP were examined for surface fluorescence using epifluorescence and phase-contrast illumination of an Olympus BH-2 microscope. Bright fluorescence of the zona surface indicated the presence of PZP specific antibodies (Plate 1). At dilutions near the endpoint oocytes with fluorescent zonas and oocytes with non-fluorescent zonas were counted (total average no = 50 oocytes). The following scores were given: +++ for 81-100% oocytes with fluorescent zonas, ++ for 61-80%, + for 31-60%, + for 6-30%. + was considered a weakly positive reaction, reactions below 5% were considered negative. The greatest serum dilution which resulted in a positive reaction was considered the anti-zona antibody titer (ZAT) of that serum.

For the IIFA with equine oocytes the latter were obtained from frozen-thawed ovaries by mincing the ovarian tissue in ice-cold PBS and filtering the mixture through gauze and a 73 µm screen. The oocytes were recovered from the cellular material trapped on the 73 µm screen. The IIFA procedure was essentially the same as performed with porcine oocytes, however, an average of only 5 equine oocytes were utilized to test each serum dilution. The presence of small numbers of equine oocytes per ovary when compared to porcine oocytes (an average of 7 oocytes per equine ovary versus 200 oocytes per porcine ovary), and the difficulty in obtaining equine ovaries did not permit large scale usage of equine oocytes per test. The oocytes were incubated with the various reagents on a Boerner slide that was placed inside a humidified chamber. Instead of being washed by centrifugation, the oocytes were repeatedly transferred to watchglasses containing PBS by means of a micropipette. All transfers were performed under a stereomicroscope.

In vitro assessment of sperm binding

Porcine oocytes were incubated in heat-inactivated fetal calf serum and washed 3 times. Then 200 oocytes were incubated with 600 microliter horse anti-PZP serum, and washed 3 times as described in the first portion of the IIFA. Non-immune and pre-immune serum served as controls. The oocyte pellet was resuspended in 30µl PBS and transferred to a 5 ml sterile culture tube containing 0.4 ml boar sperm at the concentration of 5×10^6 per ml in basal medium (BM) with 0.5% BSA at pH 7.7. The fresh sperm had been centrifuged on Percoll and capacitated by incubation in the same medium (BM with BSA) and in a humidified incubator with 5% CO₂ at 39°C for at least 3 hours (Berger, T., 1985). The oocytes were gently mixed with the sperm and incubated overnight in the same incubator.

The stop-fix technique of Saling, Storey and Wolf (1978) for recovering sperm tightly bound to zona was modified for treatment of the oocyte-sperm mixture. The mixture was carefully layered on discontinuous Dextran (500 000 MW, Sigma) gradients of 1.8% (0.35 ml) over 2.25% (0.2 ml) in BM without BSA in a 1 ml Fisher tube and centrifuged in a Fisher microfuge at approximately 100g for 2 minutes. The supernatant was discarded, the pellet was resuspended in 25µl of 2.5% glutaraldehyde in PBS and transferred onto a slide for microscopic examination. An average of 60 oocytes was counted per slide. The various degrees of sperm binding were classified in the following three categories: 1) None, no or less than 5 sperm bound per oocyte, 2) Intermediate, 5-20 sperm bound and 3) Heavy,

more than 20 sperm bound.

For statistical evaluation of the correlation of sperm binding with ZATs, the total percentage of oocytes with sperm binding (heavy and intermediate combined and heavy alone) was converted by $\text{arc sin } \sqrt{p}$.

RESULTS

Indirect immunofluorescence assay (IIFA) with porcine oocytes

Serum samples from all the horses in this study were negative for anti-zona antibodies prior to immunization. Serum samples collected from the feral mares 10 days following the first immunization resulted in ZATs of <1:100 (Table 1). Following the 3rd inoculation ZATs ranged from 1:400 to 1:1600. The mares were returned to their domicile after the fourth injection and therefore were not available for assessment of their ZATs resulting from the fourth injection. Upon recapture 8 months later, 7 of the 9 mares tested still had ZATs ranging from 1:200 to 1:1600, two mares had no detectable antibodies and one could not be recaptured.

The antibody responses of the 4 fertile mares (ST, AP, GO, SH), the infertile mare (SQ) and the gelding (AL) in the group of domestic horses, to the PZP inoculations over a period of 23 months are illustrated in Text-fig. 1. The ZATs following the first injection were not higher than 1:10. After the third inoculation the ZATs ranged from 1:200 to 1:1600 and after the fourth injection, between 1:800 and 1:6400. Within the next 4 to 9 months the ZATs among the mares in this group declined steadily to a range of 1:100 to 1:400. When their ZATs had reached low levels (ST 1:100, AP 1:200, GO 1:200 and 1:100, SH 1:400) 6-10 months later they received booster inoculations of 20000 PZP. All but one mare (GO) responded with ZATs of 1:3200. The mare GO received two booster injections 4 months apart. Both resulted in poor response.

It had been anticipated that the gelding (AL) and in particular the infertile mare with chromosomal abnormality (SQ) would have responded differently to the immunizations because they lacked follicles and had never been exposed to zona components. However, there were no observable differences in the degree of immune response in the infertile mare when compared with the fertile mares. In fact SQ's immune response and antibody level maintenance was very similar to the fertile mare GO, which had the lowest response to immunizations among the 4 fertile domestic mares. The gelding, however, maintained a good level of immunity for 11 months following the initial immunizations.

IIFA with equine oocytes

An immune serum was chosen that had been obtained from the mare AP following the third inoculation with PZP (Table 2). Two-fold dilutions were prepared from 1:5 to 1:1600 in PBS. When tested with porcine oocytes the ZAT was 1:800. The highest dilution of the same serum that resulted in a positive reaction with equine oocytes was 1:20. A lower titer had been anticipated because a heterologous antibody (serum raised from pig zona) was tested with equine oocytes.

In vivo block of fertilization

Of the 9 feral mares recaptured 8 months following the initial series of 4 injections, one mare was estimated to be approximately 21 days pregnant as determined by rectal palpation. This mare was one of the two mares found negative for PZP antibodies at that time (see Table 1). Confirmation of pregnancy by ultrasonography was not performed because of the impracticality of applying this methodology to wild mares. No detectable evidence for pregnancy by rectal palpation was noted in the remaining 8 mares. Although the 10th mare could not be recaptured there was no observable evidence to indicate that she was heavily in foal at the time of the examination of the other 9 mares. Sixteen months after the last injection, this mare had not produced an observable live foal.

Ten months following the initial series of 4 injections, 3 of the 4 fertile domestic mares (AP, GO, SH) were diagnosed 24 to 26 days pregnant by ultrasonography. At the time of estimated conception the ZATs were 1:100, 1:200 and 1:400 (Text Figure 1, C = conception). One mare (ST) was diagnosed pregnant at 22 days gestation 8 months after the booster injection when the ZAT was 1:100. All 4 fertile mares had been bred on an average of 7 estrous cycles each over a 10 month period prior to conceiving. The pregnancies of AP, GO, and SH were terminated by injections of prostaglandin. ST's pregnancy was not terminated for observation of possible side effects from the immunizations on pregnancy.

In vitro block of sperm binding

Sperm binding assays utilizing equine oocytes and sperm with the antisera described were unsuccessful due to inconsistent sperm binding controls. Therefore, a porcine assay system was utilized. Text-fig. 2 illustrates the results of the sperm binding assay using porcine oocytes and boar sperm. Sera of different antibody levels as demonstrated by the IIFA were selected from bleedings at different times over the 23 months period from the mare ST (Text-fig. 1). Their ZATs were 1:10, 800, 400, 200, 100, 100, 3200, 200, 200, 100 and they had been collected at 1, 2, 6, 8, 9, 10, 11, 17, 18 and 21 months respectively. Non-immune serum from a random mare and pre-immune serum from ST served as controls. All sera were used undiluted. 95% of the control oocytes had heavy sperm binding. The various degrees of sperm binding after incubation of the oocytes with the immune sera are arranged in order of the sera's ZATs. Sera of lower ZATs generally produced higher sperm binding rates. The only two exceptions among the 10 sera tested were the ones collected at 17 and 2 months with respective ZATs of 1:200 and 1:800. Both of the sera blocked sperm binding 4% less than sera of the next lower ZAT. Sera of identical ZATs collected at different times produced similar degrees of sperm binding. Sera with ZATs of 1:100 and 1:200 were selected to test for reproducibility because antibody concentration in this range allowed conception in vivo. Antibody titers were significantly correlated with blocking of combined heavy and intermediate sperm binding ($p=0.01$) and also with blocking of heavy sperm binding alone ($p\leq 0.05$).

A similar experiment was performed using various bleedings from the mare AP. Identical ZATs produced similar degrees of inhibition of sperm binding. Because of a lower sample size (8), their correlation was not significant ($p\leq 0.10$).

When sperm binding was tested with oocytes preincubated with two-fold dilutions (in PBS) from an antiserum with a ZAT of 1:1600 (feral mare No.3) a gradual decrease of inhibition of sperm binding was found up to dilution 1:400. Dilutions of 1:800 and above produced similar degrees of sperm binding as the zona antibody negative controls.

DISCUSSION

It is clear from these results that an immune response in horses is provoked by heteroimmunization with solubilized PZP. Other investigations confirm the effects of heteroimmunization when compared with alloimmunization (Wood, 1981; Mahi-Brown, 1982; Gwatkin, 1977), whereby heteroimmunization appears to be the more immunogenic and therefore preferable method of eliciting anti-zona antibodies. It is difficult to compare the degree and the duration of the response shown in horses with that of other mammalian species studied because the methods of assay, dosage of antigen and the size of experimental animals differ. The present study indicates that, in the horse, a relatively strong antibody response to PZP can be obtained. The effect on fertility is temporary and persists for 8 or more months in most of the animals studied.

It was unfortunate that an evaluation of the antibody effect on fertility for one continuous physiologic breeding season (March through September) following the use of PZP was not possible. Due to the time this study was initiated (June) the remaining effects on fertility were studied during the first half of the following physiologic breeding season (March to June). Because of the apparent block of *in vivo* fertilization during the second half of the first and the first half of the second physiologic breeding season, it is reasonable to assume that the presence of more than adequate levels of PZP antibodies in the mares during the anestrus period would have inhibited fertilization had the mares continued estrous cycles through this period.

In assessing possible causes for temporary inhibition of fertility following the use of PZP, ovarian malfunction and/or abnormal effects on the estrous cycle were investigated. As determined by rectal palpation and daily teasing of the mares by a stallion, the follicular development, cyclicity, ovulation and duration of the estrus of the 4 fertile domestic mares remained normal throughout the investigation.

Although endocrinological profiles or histological examination of the ovaries were not investigated, it can be stated on the basis of clinical evidence that the immunization with PZP did not cause any impairment of cyclicity or fertility. All 4 mares continued to cycle normally and became pregnant once PZP antibodies decreased to minimal levels. It appears, therefore, that inhibition of fertility occurs by some process other than ovarian malfunction. In view of the ovarian changes reported by other investigators following the use of PZP or their specific glycoproteins, the lack of clinical evidence for ovarian malfunction in this study could possibly be attributed to the proportionately lower dose of antigenic components administered. Skinner et al. (1984) reported abnormal ovarian follicular changes in rabbits after one or two injections of 300 µg of PZP proteins. According to PZP antigen purification yielding 33 ng per zona (Dunbar et al., 1980), 300 µg PZP antigen are approximately equivalent to

9000 PZP. Alterations in ovarian function were mentioned by Mahi-Brown et al. (1982); Mahi-Brown et al. (1985) after immunization of bitches with 4-6 monthly injections of 2000 and 1000 PZP. Considering the average weight of the rabbit 2.5 kg, and of the dog 17kg, the rabbits received doses of 4000 PZP per kg and the dogs received doses of 118 and 59 PZP per kg. In contrast, the horses in this study (450 kg) received only 4 or 11 PZP per kg 4 times in three months and one booster injection of 44 PZP per kg 6-10 months later.

In conjunction with the results obtained from the in vitro assessment of sperm binding, this study suggests that the cause for temporary inhibition of fertility could be due to some impairment at the level of the zona pellucida that prevents stallion sperm from binding and thus penetrating ova in the presence of anti-zona antibody. It is hypothesized that sperm binding sites on the surface of the zona may be blocked by direct antibody binding or indirectly blocked by steric hindrance from antibodies bound to other molecules in close proximity to the binding sites. In either case, sperm binding and subsequent penetration is prevented.

In the past, stallions in wild horse populations in the Northern hemisphere have been the target for temporary sterilization as a means of controlling overpopulation. These attempts have met with minimal success. The use of crude preparations of PZP may serve as an attractive alternative for the control of wild horse populations where management levels are desired. Provided a mare is fertile, the inhibition of fertilization for 1 physiologic breeding season results in the birth of 1 foal for a period of 2 years. This represents approximately one half of the normal number of foals produced annually for each mare immunized.

Further investigations are needed to determine whether the use of purified fractions of PZP would achieve higher titers and longer lasting immunity. Immunization of pregnant mares and its effect on the fetus and the mare's future fertility also need to be studied.

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REFERENCES

- Berger, T. (1985). Incubation conditions affecting ability of boar sperm to penetrate zona-free hamster ova. Annual Meeting of the American Society of Animal Science. Abstract, In Press.
- Dunbar, B.S., Wardrip, N.J. & Hedrick, J.L. (1980). Isolation, physicochemical properties, and macromolecular composition of zona pellucida from porcine oocytes. *Biochem.* 19: 356-365.
- Gwatkin, R.B.L., Williams, D.T. & Carlo, D.J. (1977). Immunization of mice with heat-solubilized hamster zonae: Production of anti-zona antibody and inhibition of fertility. *Fert. Steril.* 28: 871-877.
- Gwatkin, R.B.L. & Williams, D.T. (1978). Immunization of female rabbits with heat-solubilized bovine zonae: Production of anti-zona antibody and inhibition of fertility. *Gamete Res.* 1:19-26.
- Gwatkin, R.B.L., Andersen, O.F. & Williams D.T. (1980). Large scale isolation of bovine and pig zonae pellucidae: Chemical, immunological and receptor properties. *Gamete Res.* 3:217-231.
- Jilek, F. & Pavlok, A. (1975). Antibodies against mouse ovaries and their effect on fertilization in vitro and in vivo in the mouse. *J. Reprod. Fert.* 42: 377-380.
- Liu, I.K.M. & Shivers, C.A. (1982). Antibodies to the zona pellucida in mares. *J. Reprod. Fert., Suppl.* 32: 309-313.
- Mahi-Brown, C.A., Huang, T.T.F. & Yanagimachi, R. (1982). Infertility in bitches induced by active immunization with porcine zonae pellucidae. *J. Exp. Zool.* 222:89-95.
- Mahi-Brown, C.A., Yanagimachi, R., Hoffman, J.C., & Huang, T.T.F., Jr. (1985) Fertility control in the bitch by active immunization with porcine zonae pellucidae: Use of different adjuvants and patterns of estradiol and progesterone levels in estrous cycles. *Biol. Reprod.* 32, 761-772.
- Oikawa, T. & Yanagimachi (1975). Block of hamster fertilization by anti-ovary antibody. *J. Reprod. Fert.* 45: 487-494.
- Sacco, A.G., Subramanian, M.G., Yurewicz, E.C., DeMayo, F.J. & Dukelow, W.R. (1983). Heteroimmunization of squirrel monkeys (*Saimiri sciureus*) with a purified porcine zona antigen (PPZA): immune response and biologic activity of antiserum. *Fert. Steril.* 39: 350-358.
- Saling, P.M., Storey, B.T. & Wolf, D.P. (1978). Calcium-dependent binding of mouse epididymal spermatozoa to the zona pellucida. *Devl. Biol.* 65: 515-525.
- Shahani, S.K., Padbidri, J.R. & Rao S.S. (1972). Immunobiological studies with the reproductive organs, adrenals and spleen of the female mouse. *Int. J. Fertil.* 17: 161-165.

Shivers, C.A., Sieg, P.M. & Kitchen, H. (1981). Pregnancy prevention in the dog: Potential for an immunological approach. J. Am. Anim. Hosp. Assoc. 17: 823-828.

Shivers, C.A. & Liu, I.K.M. (1982). Inhibition of sperm binding to porcine ova by antibodies to equine zonae pellucidae. J. Reprod. Fert., Suppl. 32: 315-318.

Skinner, S.M., Mills, T., Kirchick, H.J. & Dunbar, B.S. (1984). Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion. Endocrinology 115: 2418-2432.

Tsunoda, Y. & Chang, M.C. (1976). In vivo and in vitro fertilization of hamster, rat and mouse eggs after treatment with anti-hamster ovary antiserum. J. Exp. Zool. 195: 409-416.

Wood, D.M., Liu, C. & Dunbar, B.S. (1981). Effect of alloimmunization and heteroimmunization with zonae pellucidae on fertility in rabbits. Biol. Reprod. 25: 439-450.

TABLE 1

Antibody titers to porcine zona pellucida in feral horses

	Preinoc.	10 days after 1. inj. of 5000 PZP	1 month after 2. inj. of 2000 PZP	1 month after 3. inj. of 5000 PZP	10 months after 4. inj. of 5000 PZP	Findings from rectal palpation ^a
1	neg	1:10	1:100	1:800	1:400	not preg.
2	neg	1:10	1:100	1:800	1:400	not preg.
3	neg	1:10	1:100	1:1600	1:400	not preg.
4	neg	1:10	1:100	1:800	neg	not preg.
5	neg	1:10	1:100	1:400	1:400	not preg.
6	neg	neg	1:100	1:800	NDb	(not preg) ^c
7	neg	1:10	1:100	1:400	1:200	not preg.
8	neg	1:10	1:100	1:400	1:400	not preg.
9	neg	1:100	1:100	1:800	1:1600	not preg.
10	neg	1:100	1:100	1:800	neg	pregnant ^d

a = Findings from rectal palpation done 8 months following the 4th injection

b = Not done because the mare could not be caught

c = Not pregnant as based on observation from a distance

d = Approximately 21 days pregnant as estimated by rectal palpation

Antibody titers as found in the indirect immune fluorescence assay, from bleedings obtained from the feral mares after the 4 initial immunizations. Findings from the rectal palpation performed when the mares were recaptured 8 months after the 4th immunization, are also included for comparison with their respective antibody levels. Note that one of the 2 mares with a negative titer was found pregnant.

TABLE 2

Comparison of antibody titers of equine anti-PZP serum
using porcine and equine oocytes.

Serum	Dilution In PBS	% Oocytes with Fluorescent Zonas	
		Porcine	Equine
Pre-immune ^a	1:5	0 ^b	0 (4) ^c
After Third Inoculation ^a	1:5	100	100 (6)
	1:10	100	80 (5)
	1:20	100	20 (5)
	1:50	100	0 (5)
	1:100	100	ND ^d
	1:200	100	0 (3)
	1:400	82	0 (4)
	1:800	29	0 (4)
	1:1600	0	0 (4)

^a Equine preimmune and anti-PZP serum (after third inoculation) were obtained from the mare AP.

^b 50 Porcine oocytes counted per dilution

^c Number of equine oocytes counted per dilution

^d Not done

Legend to Text-fig. 1

Endpoint immunofluorescent antibody levels in sera obtained over a period of 23 months from domestic horses after immunization with solubilized PZP. ST, AP, GO and SH are fertile mares, SQ is an infertile mare with a chromosome abnormality (63x0 genotype) and AL is a gelding.

↓ = inoculation

C = conception

Legend to Text-fig. 2

Varying degrees of boar sperm binding on porcine oocytes preincubated in undiluted anti-sera of different antibody titers to porcine zona from the mare ST.

Sperm binding is expressed in % oocytes with heavy =  , intermediate =  and negative =  binding and is illustrated in order of the sera's antibody titers. Controls consist of serum from a non-immunized mare and pre-immune serum from the mare ST.

- a The month serum was collected during the 23 months study. Month number corresponds to Text-fig. 1.
- b Reciprocal antibody titer to porcine zona.

PLATE 1

Fig. 1. IIFA treated porcine oocytes under phase contrast illumination: incubated with preimmune (left) and immune (right) serum from a mare. The oocyte treated with immune serum shows a heavy precipitate on the zona surface. Approximately x 530.

Fig. 2. Same oocytes as shown in Fig. 1 under epifluorescence illumination: oocyte without fluorescence of zona surface (left) and oocyte with brilliant fluorescence of the zona surface indicating bound anti-zona antibody (right).

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