

## EFFECTS OF IMMUNIZATION WITH PORCINE ZONA PELLUCIDA AND OOCYTES UPON REPRODUCTIVE FUNCTION IN THE BITCH

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*In the last two decades various ways for preventing pregnancy have been investigated, and in this respect, a reversible influence on reproductive function exerted by drugs without induction of side effects, still remains undiscovered. Recently we recognized that immunological agents directed against mammalian gametes could inhibit fertilization. Among them, antibodies against zona pellucida (ZP) are a very promising tool for supervising the process of reproduction in domestic animals.*

*In the present work, we have investigated the effect of administration of porcine zona pellucida alone (ZPp), or together with its oocyte (OV+ZPp), on reproductive function in the bitch. Bitch immunization consisted of 6 consecutive injections at 3 week intervals and each dose contained 500 ZPp (alone or together with their oocytes). In the first two bitches immunized with ZPp, high titres of antibodies suppressed fertilization, for 7 months, but exerted no effect upon sexual cyclicity. Bitches that were immunized with OV+ZPp, also developed high titres of antibodies, suppressive for fertilization and lasting for a longer period (10 months). However, these animals had reproductive disorders. Both immunoconceptive methods were 100% effective.*

*Key words: immunocontraception, zona pellucida, bitch*

### INTRODUCTION

The zona pellucida (ZP) plays an important role in fecundation. In mammals, the ZP glycoprotein mediates essential events during fertilization, regarding the initial attachment of sperm to ZP and also for the acrosome reaction induced by ZP (Hafez, 1993, Jewengow et al. 1998., Wassarman, 1998, Strom Holst, 1999).

For a long time the ZP antigens have been considered as interesting targets for immunocontraception because antibodies directed against ZP are tissue specific and can prevent fertilization (Porter, 1965, Mahi and Yanagimachi - 1976, Paterson et al. 1998).

Two major events that can be blocked by antibodies against ZP are sperm attachment to ZP receptors for initiating fertilization and embryo hatching before implantation. Antibodies (anti-ZP) from the follicular fluid of the immunized bitches, linked to immune complexes from the surface of oocytes in the ovary of the same bitch suggests that these antibodies form complexes with the ZP antigens of the oocytes. According to that, infertility might be generated through the blockade of sperm attachment. The immune complexes could hide the receptors for sperm cells or could even, mechanically prevent sperm attachment. This was documented in some *in vitro* and *in vivo* studies (Mahi-Brown et al., 1992).

The duration of the contraceptive effect, following active immunization, depends on the serum and follicular antibody levels and also on the time period necessary for their disappearance from the ovaries and oocytes, in the case of passive immunization. The contraceptive effect disappeared when the antibody titre decreased and when the coated oocytes disappeared from the ovaries, because of ovulation or atresia.

ZP is a weak alloantigen but a strong xenoantigen and so a better immune response was obtained when ZP of one species was used for immunization of females of another species. In many cases, xenogenic antizona-antibodies reacted with ZP of immunized animals (Mahi-Brown et al., 1988, Jewengow et al. 1998, Wegner et al. 1998).

The aim of this study was to establish a titre of antibodies to ZPp or OV+ZPp in immunized bitches and to evaluate their contraceptive effect. Furthermore, we wanted to find out if these antibodies alter sexual behaviour in the immunized females.

#### MATERIAL AND METHODS

For this investigation we used 4 mongrel bitches, weighing between 12 and 15 kg. Initially they were clinically examined, including abdominal and pelvic ultrasonography for excluding pregnancy. All animals were kept in separate kennels, with water *ad libitum* and fed once a day in the morning with commercial dog food. Twice a week, a cytovaginal smear from each animal was examined for estrus detection.

**Antigen preparation.** Sow ovaries were obtained from the local slaughterhouse and only those with normal macroscopic appearance (without cysts) were used. The ovaries were frozen until processed, because freezing does not affect the antigenic properties (Mahi and Yanagimachi, 1978, Shivers et al., 1981). Although Mahi and Yanagimachi (1976, 1978), Gwatkin et al. (1980) and Henderson et al. (1988) described a very simple and rapid method for obtaining oocytes from sow ovaries, the lack of some basic elements such as nylon screens with mesh sizes varying between 100, 136 and 215 micrometers (Shivers et al. 1981) determined us to aspirate the liquid from each follicle on the ovary surface and to flush it into a Petri dish containing TCM 199 (Tissue Culture Medium 199, Sigma). Following that, each oocyte surrounded by ZP was aspirated using mouth-operated micropipettes, under a dissecting microscope. We were able to isolate an average of 25 oocytes from each ovary. Oocyte denudation was accomplished by keeping them in 0.3% collagenase solution, for 60 minutes at 37 °C. The ZP fragments were harvested by centrifugation (5 min., 3000 rpm, 4 times) and resuspended in TCM. Single doses for immunization

consisted of 500 oocytes with their ZP or ZP alone (obtained from 500 oocytes). When only ZP was used for immunization it was previously solubilized by heating for 45-60 minutes at 70 °C (Gwatkin et al., 1980, Mahi-Brown et al., 1985). Solubilization was confirmed by observation under the dissecting microscope. Each immunization dose was resuspended in 2 ml of TCM 199 (Tissue Culture Medium, Sigma) and frozen in plastic tubes. The titer of antibodies was established using the slow agglutination test (Coombs 1964).

Bitch immunization consisted of 6 consecutive injections at 3 week intervals, as follows: bitches A and B were immunized with porcine ZP (one dose consisted of ZP obtained from 500 oocytes) and bitches C and D were immunized with material that consisted of 500 sow oocytes together with their ZP (OV+ZPp). Before administration, each dose was thawed and mixed with an equal volume (2 ml) of sterile Al(OH)<sub>3</sub>. The dose was subcutaneously administered on the lateral part of the neck, after 5 minutes of stabilization at room temperature.

Blood samples were obtained from the antebrachial vein before the first immunization and at 14 day intervals after each immunization. Starting from the 6th month after the first immunization samples were collected every 30 days, till the moment when no antibodies could be detected. Blood was allowed to clot at room temperature and sera samples were frozen at - 20 °C till used.

When vulvar discharges appeared, vaginal smears were prepared every 48 hours and stained by the May Grunwald Giemsa (MGG) method. Mating was allowed upon the analyses of the cytovaginal smears, twice every day, as long as the bitch accepted the male. After vaginal discharges stopped, smears were prepared twice a week. In this experiment we used two mongrel male dogs with proven fertility.

## RESULTS AND DISCUSSION

Although Shivers et al. (1981) carried out 3 consecutive immunizations at 7 day intervals for immunocontraception in bitches (each dose consisted of 2000 ZPp suspended in Freund's adjuvant) we have modified this procedure as described above (500 ZP in Al(OH)<sub>3</sub>) with satisfactory results. It is well known that immunization with ZP without adjuvant does not elicit any detectable immune response (Mahi-Brown et al., 1985)

Data obtained from the serum analyses of bitches A and B (immunized with only ZPp) are presented in Table 1. and Figure 1.

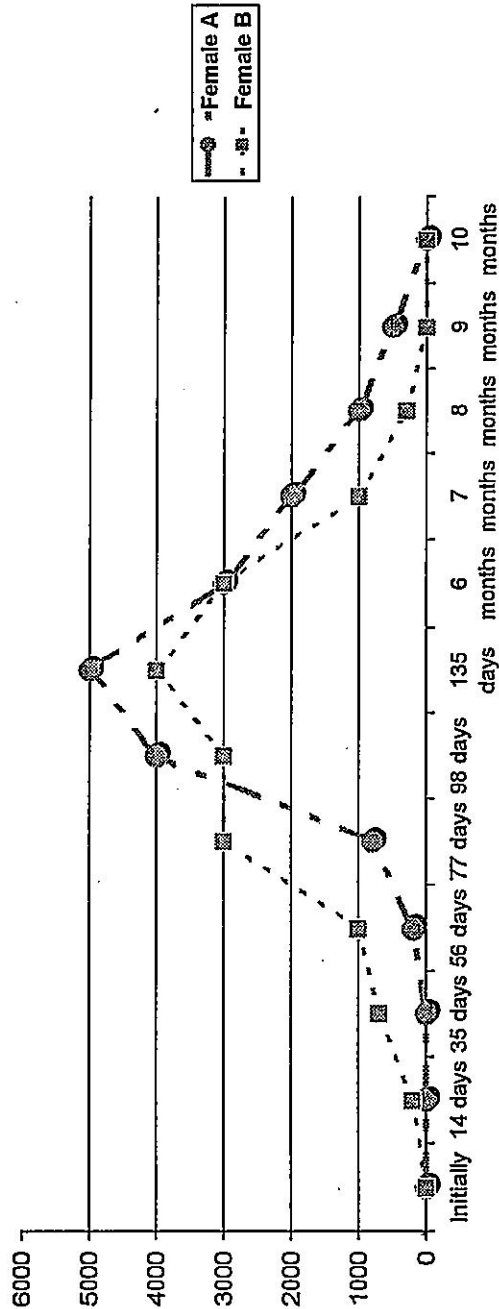
In bitch A, the first positive result (titre 1:200) was detected after the fifth immunization. This is in agreement with the results of Mahi-Brown et al. (1982) who stated that for a titre of 1:1000, sufficient for inhibition of spermatozoa penetration, 5 booster injections were necessary. The titres of anti-ZP antibodies remained above that level for 8 months, but 10 months after the first immunization, antibodies were not detectable. This female entered into estrus at 5.5 months from the first immunization and the vaginal bleeding lasted 17 days. The bitch was mounted for 3 consecutive days (12, 13 and 14) but did not become pregnant (Table 3). The vaginal smears stained by the MGG method revealed normal dynamics of the estrus cycle.

The second bitch (B) reacted with synthesis of anti-ZP antibodies after the first immunization. Their titre increased continuously till the 6th immunization reaching a maximum value of 1:4000. The antibody titre attained the level of 1:1000

**Table1. Data from bitches immunized with ZPp**

|        |                | Titer of antiZP antibodies at an interval of : |         |         |         |         |          |          |          |          |          |           |  |
|--------|----------------|--|---------|---------|---------|---------|----------|----------|----------|----------|----------|-----------|--|
| Female | Initial moment | 14 days  | 35 days | 56 days | 77 days | 98 days | 135 days | 6 months | 7 months | 8 months | 9 months | 10 months |  |
| A      | 0              | 0  | 0       | 1:200   | 1:800   | 1:4000  | 1:5000   | 1:3000   | 1:2000   | 1:1000   | 1:500    | 0         |  |
| B      | 0              | 1:200  | 1:700   | 1:1000  | 1:3000  | 1:3000  | 1:4000   | 1:3000   | 1:1000   | 1:300    | 0        | 0         |  |

**Figure 1. Titers of antiZP antibodies in the females immunized with ZPp**



after three boosters, in contrast to bitch A (where five boosters were necessary). Antibody titre remained at an inhibitory level for spermatozoa for 7 months after the first immunization (30 days less than female A) as shown in Figure 1. Bitch "B" entered the estrus cycle 6 months after the first immunization. Vaginal discharges lasted for 14 days and four matings were allowed on days 11 and 12 of the cycle. This female was very close to the average physiological values for the length of the estrus cycle and also for the mating moment. Evaluation of the cytovaginal smears did not reveal any pathological changes. After mating, this bitch did not become pregnant either.

In a study carried out in 1985, Mahi-Brown et al. obtained anti - ZP antibody titres ranging between 1:1000 and 1:4000 by 3 consecutiv immunizations at 30 day intervals. Each dose consisted of 2000 ZPp.

In another study Mahi-Brown et al (1982),. reported a maximal titre of 1:100,000 after 6 consecutive immunizations with 2000 ZPp / dose at 30 day intervals.

Data obtained from bitches C and D that were immunized with OV+ZPp using the previously described method are presented in Table 2 and Figure 2.

In female "C", the titre of antibodies sufficient for fertilization inhibition, appeared after the second immunization and reached the maximum value (1:7000) following the fifth immunization. It is important to emphasize that in this case a suppressive titre lasted for 11 months from the first immunization and that antibodies disappeared from the blood serum after 14 months. Along this entire period, the female did not cycle, as the cytovaginal smears confirmed. Starting from the 6th month after the first immunization, erythrocytes were present in the vaginal smears in a relative high number and this phenomenon lasted till the 10th month. The other elements in the smear permanently indicated that this female was in the phase of metestrus.

Female "D" reacted by antibody production after the third immunization, reaching the suppressive level after the fourth injection of antigen. The maximal titre of antibodies (1:6000) was recorded after five booster injections. Antibodies were not detectable in the blood serum after 11 months. The suppressive antibody level lasted till the 9th month. This female showed signs of estrus 8 months from the first immunization and vulvo-vaginal discharge lasted 28 days. The cytovaginal smear revealed a large amount of erythrocytes even at 7 days after discharge disappearance. There were only a few basophil cells, and we noted an ovulation disorder.

Using non purified ZPp, Mahi-Brown et al. (1985) obtained titres of anti - ZP antibodies up to 1:20.000, but also abnormal cycling in all 3 bitches included in the study.

Figure 3 presents a general view of the results in our study. In the case of immunization with ZPp, the suppressive titre of antibodies (average value 1:1900) was established after the fifth immunization and remained at this level for seven months after initiation of the protocol. When OV+ZPp were used for immunization, the suppressive titre appeared after the second immunization (average value 1:1100) and stayed above the suppressive level for 10 months after the first immunization.

In the first two bitches (immunization with ZPp), estrus appeared at an average interval of 5.5 months after the first immunization, compared to the second two animals (immunization with OV+ZPp), when estrus appeared in one bitch

Table 2. Data from bitches immunized with OV+ZPP

| Female | Titer of antiZP antibodies at an interval of: |         |         |         |         |         |          |        |        |        |        |        |
|--------|---|---------|---------|---------|---------|---------|----------|--------|--------|--------|--------|--------|
|        | Initially                                     | 14 days | 35 days | 56 days | 77 days | 98 days | 135 days | 6 mo.  | 7 mo.  | 8 mo.  | 9 mo.  | 10 mo. |
| C      | 0   | 1:800   | 1:2000  | 1:3000  | 1:5000  | 1:7000  | 1:8000   | 1:5000 | 1:4000 | 1:3000 | 1:3000 | 1:2000 |
| D      | 0   | 0       | 1:200   | 1:800   | 1:2000  | 1:3000  | 1:6000   | 1:4000 | 1:4000 | 1:3000 | 1:1000 | 1:200  |

Figure 2. The antibody titers in the bitches immunized with OV+ZPP

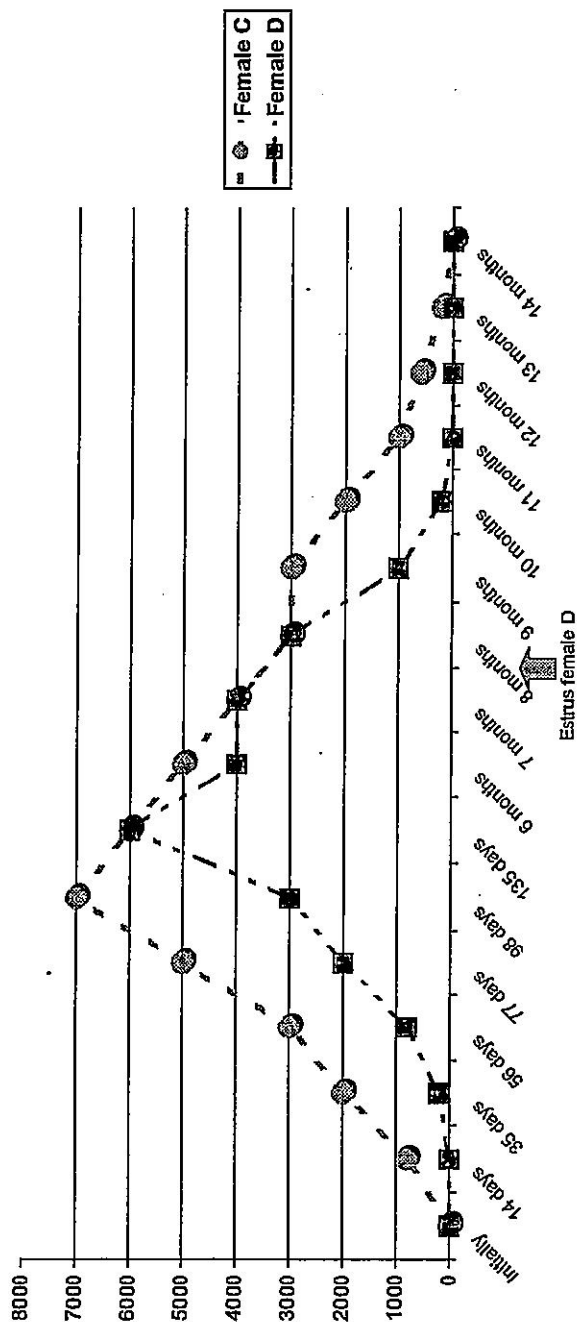


Table 3. Characteristics of the sexual activity in immunized bitches

| Female | Estrus appearance reported to 1 <sup>st</sup> immunization | Duration of vulvo-vaginal discharge | No. of mating s | Pregnancy | Sexual cycle |
|--------|--|-------------------------------------|-----------------|-----------|--------------|
| A      | 5.5 months   | 17 days                             | 6               | none      | normal       |
| B      | 6 months   | 14 days                             | 4               | none      | normal       |
| C      | not in estrus for 14 months                                | -                                   | -               | -         | abnormal     |
| D      | 8 months   | 28 days                             | 9               | none      | abnormal     |

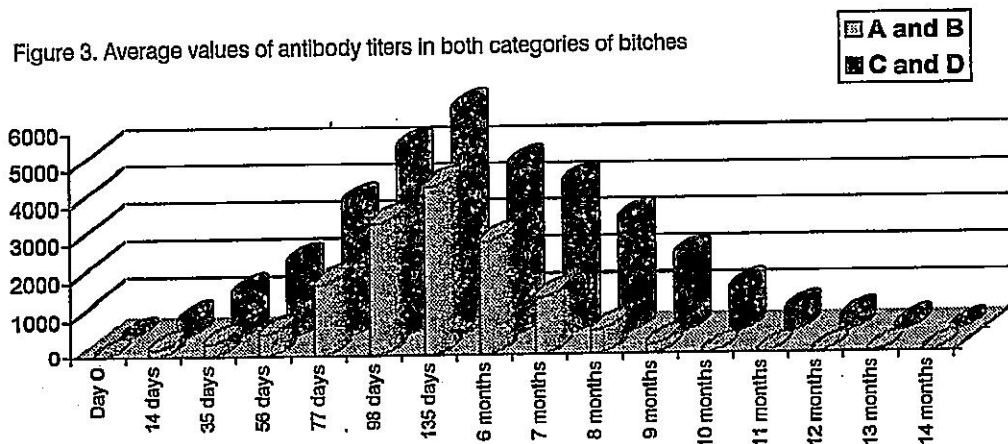
after eight months from treatment initiation, whereas in the second bitch, estrus did not appear for 14 months (while she was kept under observation)

Our results are in agreement with Mahi-Brown et al. (1988), who stated that although bitches immunized with ZPp raise antibodies suppressive for in vitro fertilization, fertilization inhibition seems not to be the only cause for infertility. Other reasons may be abnormal cycles, absence of ovulation and extensive destruction of oocytes, the last perhaps generating irreversible infertility.

Yurewicz et al. (1983) assumed that the apparent disorders in ovarian function in the immunized bitches indicate that induced antibodies are not necessarily specific for zona pellucida. This fact and also the fact that the antibodies were produced as an answer to ovarian antigens different from those of zona pellucida, could explain the elevated titres observed in bitches "C" and "D", compared to animals "A" and "B" in our study,

Skinner et al. (1983) reported a significant decrease in the number of primary, secondary and tertiary follicles in does, 6 weeks from the last immunization with ZP. After 20 weeks only a few (if any) growing follicles could be observed, so it was suggested that antiZP antibodies reacted with cells responsible for zona pellucida synthesis (Skinner et al 1983.)

Figure 3. Average values of antibody titers in both categories of bitches





## CONCLUSIONS

Immunization of bitches with porcine zona pellucida generated a titre of antibodies suppressive for fertilization, persisting for 7 months, and exerting no effect upon sexual cyclicity. When oocytes, together with the zona pellucida were used for immunization, high titres of antibodies suppressive for fertilization lasted for a longer period (10 months) but were accompanied by reproductive disorders. Both immunocontraceptive methods were 100% effective within the mentioned periods.

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## **EFEKTI IMUNIZACIJE KUJA ZONOM PELUCIDOM I OVOCITIMA KRMAČA NA NJIHOVE REPRODUKTIVNE FUNKCIJE**

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### **SADRŽAJ**

U ovom radu su opisani efekti imunizacije kuja zonom pelucidom i/ili ovocitima krmača na njihove reproduktivne funkcije. Imunizacija kuja je izvedena sa šest uzastopnih injekcija koje su sadržavale po 500 zona pelucida (sa ili bez ovocita) u intervalima od 3 nedelje. Kod dve kuje, imunizovane samo zonom pelucidom, visok titar antitela je blokirao fertilizaciju i zadržao se 7 meseci bez uticaja na pojavu seksualnih ciklusa. Kod druge dve kuje, imunizovane mešavinom zone pelucide i ovocita, takođe su nastala antitela koja sprečavaju fertilizaciju i njihov visok titar se održao 10 meseci. Međutim, kod ovih životinja su registrovani poremećaji estralnog ciklusa. Obe kontraceptivne metode su bile 100% efikasne.

