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THE INFLUENCE OF IMMUNIZATION WITH PORCINE ZONA PELLUCIDA UPON BITCH OVARIES

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The xeno-immunization potentials of zona pellucida (ZP) and their effects on reproductive functions are supported by numerous experimental data. The variations in immune response depend on the immunogenic structure (fractions of purified or non purified zona pellucida) affecting both ovarian structure and function.

In the present study, we have investigated the effect exerted by a different number of immunizations with porcine ZP on canine oocyte capacity to resume and undergo meiosis, and also on the histological structure of ovaries in the immunized bitches. Immunization and boosters were performed with equal doses of porcine ZP together with oocytes (one administration in group B, two in group C and three in group D). Following ovariectomy, oocyte development in vitro was estimated and histological sections examined for the presence of histo-pathological changes. The increasing number of boosters negatively influenced oocyte capacity for resuming and undergoing meiosis. Furthermore, alterations of ovarian structure were more prominent and were manifested by the absence of normal ovarian follicles and oocyte loss.

Key words: immunocontraception, meiotic competence, ovarian structure.

INTRODUCTION

Control of the population size in some animal species has always been a challenge for reproductive biology and recently for molecular biology as well. Considering the molecular basis of sperm-egg interaction (Kerr *et al.*, 1998; Naz *et al.*, 2000) various approaches have been employed in order to develop immunocontraceptive strategies based on sperm antigens, *zona pellucida* antigens or hormones as antigens (reviewed by Feng *et al.*, 1999). In many studies it was demonstrated that the antigenic capacity of zona pellucida (ZP) proteins is very complex and that antibodies against ZP antigens might influence ovarian development. The immune response depends on both ZP immunogenicity and the species immunized (Skinner *et al.*, 1983, 1999,

Mahi-Brown *et al.*, 1992). For example, immunization of rabbits with porcine ZP leads to infertility and complete ovarian disgenesis, while immunization of mice and rats with the same antigens has no effect upon fertilization, although circulating antibodies recognizing ZP are detected.

Immunisation with some ZP proteins may induce an immune response that affects normal follicular development. Immunisation of monkeys and rabbits with porcine ZP3a decreased fertilisation without affecting ovarian functions, while porcine ZP3b (mouse ZP3 homologue) resulted in ovarian disgenesis.

Trying to explain how contraceptive vaccines induce ovarian pathology, both Mahi -Brown *et al.* (1992) and Paterson *et al.* (1996) investigated ZP3 antigens starting from the assumption that active immunisation against ZP antigens induces infertility by an interruption (mediated by antibodies) of sperm cell-oocyte interaction, and also disturbs ovarian functionality, because certain epitops on ZP3 generate histo-pathological effects. A peptide consisting of seven amino acids (AA, 336-343) was later isolated representing a permanent epitope with a potential to suppress fertility and also to induce oophoritis. The study of Sun *et al.* (1999) showed that female mice immunised with a B cell epitope of sulphated glycoprotein ZP2 reduced litter size but no oophoritis was observed.

It was also emphasised that active immunisation with ZP proteins led to the depletion of primordial follicles, through a still not clear mechanism (Brandon *et al.*, 1998). Small quantities of ZP glycoproteins present on the primordial follicles and granulosa cells are probably one of the reasons for pathological changes of the ovarian structure (Bagavant *et al.*, 1997, Paterson *et al.*, 1999).

The aim of this study was to investigate the effects of different numbers of immunizations (boosters) with porcine ZP. We determined the influence of anti-ZP antibodies on canine oocyte capacity to resume and undergo meiosis and also the histological changes in ovarian structure. A successful immunocontraceptive vaccine could help in solving the problem of the stray dog population.

MATERIAL AND METHODS

In this study twelve mongrel bitches were used weighing from 12 to 15 kg. They were clinically examined, including abdominal ultrasonography to exclude pregnancy or oestrus. The animals were kept in separate kennels, with water *ad libitum* and fed once a day with a commercial dog food. Twice a week, a cytovaginal smear from each animal was examined for oestrus detection.

Antigen preparation: This was performed according to the method described earlier (Mircu *et al.*, 2000). Briefly, fluid from each follicle on the ovary surface was aspirated and then flushed into a Petri dish containing TCM 199 medium (Sigma). Following that, each oocyte (surrounded with ZP) was aspirated under a dissecting microscope. We were able to isolate an average of 25 oocytes from each sow ovary. Oocyte denudation was accomplished by keeping them in 3% collagenase solution, for 60 minutes at 37 °C. The ZP fragments were collected by centrifugation (5 min., 3000 rpm) and resuspended in TCM 199. Each dose consisting of 500 oocytes with their ZP proteins, was suspended in 2ml of TCM 199 and frozen (-20 °C) in plastic tubes.

Immunisation procedure: Each group of animals consisted of three bitches and they were immunized as follows: group B was immunized with a single porcine oocyte - ZP dose, group C with two doses given at a 21 day interval and

group D with three doses, administered at 3 week intervals. Before administration, each dose was mixed with an equal volume (2ml) of sterile $Al(OH)_3$ preparation because ZP without an adjuvant does not generate any immune response (Mahi - Brown *et al.* 1985) and allowed to stabilize for 5 minutes at room temperature. The dose was subcutaneously injected into the lateral part of the neck. Bitches belonging to group A were used as control animals and were subjected to ovariectomy at the end of the experiment. Females from groups B, C, D, were ovariectomised 3 weeks after the last immunization.

Oocyte development in vitro: After ovariectomy, ovaries were placed in physiological saline at 38°C. After a maximum of 30 minutes they were punctured with a needle (20 Gauge), follicular fluid was collected in a Petri dish and observed under a binocular microscope. With the help of a micropipette the oocytes were collected and then washed successively (3 times) in PBS medium. Then they were washed in two medium baths with TCM 199 and 10% foetal calf serum (FCS).

The obtained material was placed in a NUNK dish with 4 buckets and kept in an incubator (NAPCO 5400 type) at 38.9°C for 48 hours, in air saturated with water vapour. The incubation interval was set to correspond with the interval that the oocyte naturally spends in the oviduct, for resuming and undergoing meiosis.

After incubation, oocytes were hypotonized with 4% sodium citrate solution, preserved in acetic acid : glycerol for 2-3 minutes and fixed in acetic acid: formaldehyde for 24 hours. Later on, cells were stained with acetic aceto-orscein, to examine the nucleus (Hewitt, 1997).

Histological sections: Ovaries were fixed in formaline solution and prepared according to the standard procedure prior to haematoxylin-eosin staining.

RESULTS AND DISCUSSION

Unlike the majority of mammalian species in which oocyte maturation is completed even before ovulation, the canine oocyte is ovulated as an immature or primary oocyte, achieving meiotic maturation inside the oviduct.

Oocyte capacity to undergo meiosis in suitable conditions is defined as meiotic competence (CM). The capacity of an oocyte to undergo GBV (Germinal Vesicle Breakdown) can be used as the basis for establishing meiotic competence because its appearance is easy to notice, but, on the other hand the completion of nuclear maturation at the second metaphase is probably a more precise measure. The oocyte first obtains the capacity to undergo GVB, then to enter the first metaphase stadium and later shows the ability to move from the first to second metaphase.

In this study, after they were introduced in TCM medium from Petri dishes, the oocytes, together with surrounding cumulus cells (COC - cumulus oocyte complexes), were classified using precise criteria, as follows:

- I degree - COC - intensively pigmented and entirely surrounded by one or more layers of cumulus cells

- II degree COC - easily pigmented, partly surrounded by one or more layers of cumulus cells

- III degree COC - coloured but pale, often in atypical form, without cumulus cells.

For *in vitro* maturation only first degree COC were used while second and third degree COC were removed and considered degenerated. The number of different COC samples is presented in Table1:.

Table 1. Total number of COC collected from the females involved in this study.

Group	Total number of COC	COC 1st degree		COC 2nd degree		COC 3rd degree	
		n	%	n	%	n	%
A	27	19	70.37	3	11.11	5	18.5
B	32	20	62.5	4	12.5	7	21.87
C	22	12	54.5	5	22.72	5	22.72
D	8	0	0	3	37.5	5	62.5

It is evident that the total number of recovered COC decreased, as well as their quality, after in vitro cultivation, as the number of immunisations increased. After only one immunisation, COC I represented 62.5% of the total COC, but after three immunisations no cumulus oocytes complexes of this type were identified (Table 1).

On the contrary, after one immunisation, COC III represented 21.5% of the total COC but 62.5% after three immunisations. These changes confirmed the role of ZP antigens in follicular growth and development, indicating cross reactivity between porcine ZP antigens, canine ZP antigens, canine ZP and antigens of adjacent follicular structures. This is in agreement with Mahi-Brown *et al.* (1992), who also suggested that an immune response to ZP antigens may interrupt follicular development.

After cultivation *in vitro*, canine oocytes were stained with acetic orscein in order to estimate their meiotic competence. Meiotic competence is a two stage process. In the first stage, oocytes gain the capacity to resume meiosis or to go into the process of germinal vesicle breakdown. In the second stage they achieve the ability to complete their maturation till the second metaphase.

Depending on the degree of chromatin condensation and nuclear vesicle disappearance, oocytes were classified as oocytes resuming meiosis and undergoing the process of germinal vesicle breakdown. After completion of bivalent formation, oocytes were classified as passing the first metaphase (MI). The first anaphase stage (AI) was characterised by the appearance of two chromosome groups, achieved by chromosome pair pushing as well as by their movement at the end of the meiotic arc. The second metaphase (MII) was characterised by compact chromosome groups in the shape of the first polar globule and another prevalent group that allows individual chromosome identification.

The data concerning meiotic competence are presented in Table 2 (meiosis resumption and presence of germinal vesicle, as well as meiosis completion, namely MI, AI and MII).

Table 2. Meiotic ability of the oocytes from the four groups of experimental animals

Group	No. COC1	GVB		MI		AI		MII		Uninden-tified	
		n	%	n	%	n	%	n	%	n	%
A	19	5	26.3	4	21	3	15.7	6	31.5	1	5.26
B	20	6	30	6	30	4	20	2	10	2	10
C	12	3	25	2	16.6	2	16.6	-	-	5	41.6
D	0	-	-	-	-	-	-	-	-	0	-

There was a decrease of meiotic ability as the number of booster immunisations increased. Oocytes of females from group B (one immunisation) acquired meiotic competence in 90% of cases (30% for VG, 30% - MI, 20% - AI and 10% - MII) and only 10% were not classifiable. The results were completely different in females from group D (three immunisations) where isolation of COC complexes able to proceed through the stages needed for *in vitro* maturation was not possible.

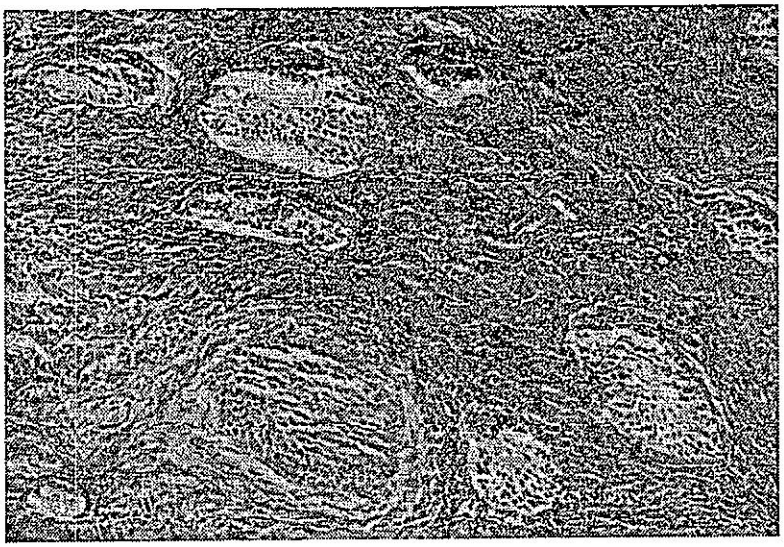

Figure 1. Reduced size of primordial follicles and absence of developing follicles in the ovary of an immunised bitch

In bitches from group D, because only II and III degree COCs were obtained, and they did not possess the necessary structures for entering into further stages, *in vitro* maturation potential was excluded. It must be noted that in group C (two immunisations), 58.2% of the oocytes were able to gain meiotic competence, but even this was incomplete, because these oocytes do not reach the MII stage. At the same time 41.7% of oocytes in this group were unclassifiable.

In the opinion of Mahi - Brown *et al.* (1982), changes induced by immunisation of bitches with ZP antigens are responsible for the absence of normal oocyte interactions with follicular fluid, interfering with the follicular growth and function. It is also possible that the generated antibodies bind to ZP and interfere with ovarian cells, which can be defined as an auto - immune attack.

Mahi-Brown *et al.* (1985) demonstrated that ZP antigens are synthesised either by oocytes, or oocytes and follicular cells. Immunoglobulins coupling to ZP antigens may induce oocyte destruction. If follicular cells secrete or phagocytize ZP material at any moment of oocyte and follicle growth, they may also become vulnerable to antizona antibodies. As a final result, their death can lead to oocyte death.

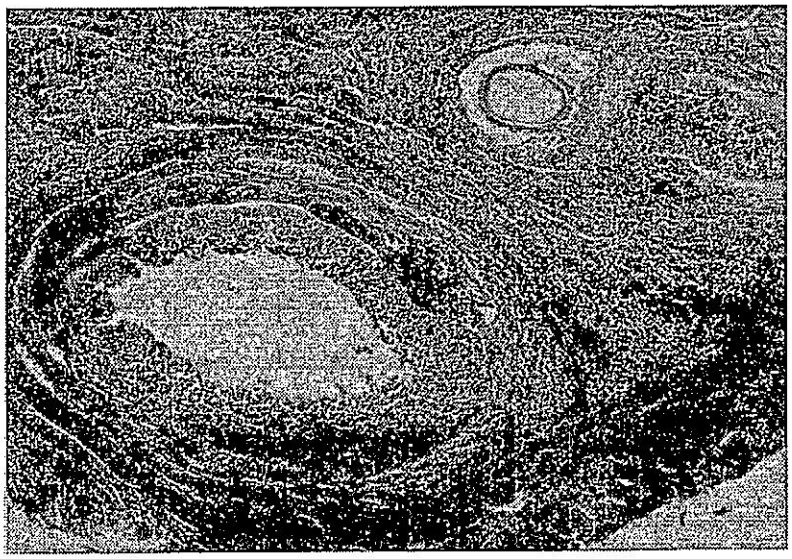

Figure 2. Thick walled cysts in the ovary of an immunised bitch

In the ovaries from the females from group D, the dominant histo-pathological finding was oocyte loss (Figure 6) and haemorrhagic infiltration (Figure 3). Oocytes could not be seen in any developing follicle, above the stage of primordial follicle, and even primordial follicles had reduced sizes. We were also able to observe thick walled cysts (Figure 2), that is in agreement with Mahi-Brown *et al.* (1988). These authors did not find lymphocyte infiltration or other signs of an inflammatory process generating the loss of oocytes.

Oocyte loss was not a consistent finding in the ovaries from the females of groups B and C (Figure 1) and no changes were noticed in the ovarian structures

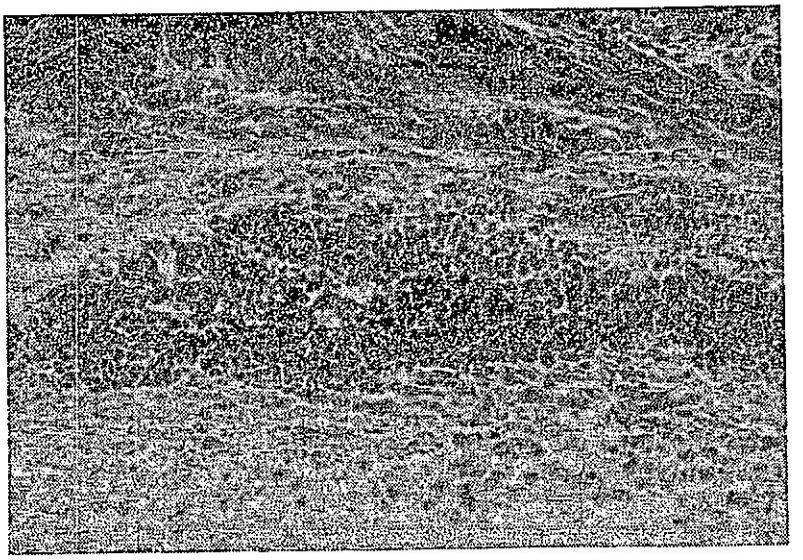

Figure 3. Haemorrhagic infiltration in the ovary of an immunised bitch

of bitches from group A. In a study designed for the use of porcine ZP vaccine in female dogs, to render the ovaries permanently inactive. Brandon *et al.* (1998) reported that, the integrity of all follicles was breached and no immunodetectable zona material was visible on sections of the ovaries after two boosters with 200mg porcine ZP in RIBI adjuvant

Variability in the immune response might be a consequence of the different genetic background (Mahi-Brown *et al.*, 1988) knowing that the immune response to antigens is under genetic control. Some of the females may have responded to one set of epitopes from ZP, while others responded to another.

The appearance of trophic disorders that generated either atresia or cyst formation seems to be induced by antibodies generated against ovarian components. The coupling of these antigens may be one of the mechanisms that interrupts trophic exchange. although, no inflammatory reaction was observed, these ovarian effects may be classified as autoimmune effects.

Lou *et al.* (1996) used a mouse model in order to investigate the mechanism of ovarian pathology induced by active immunisation with a 13 unit polymeric peptide derived from mouse ZP3 ($mZP3^{330-342}$). Active immunisation with $MZP3^{330-342}$ generated autoimmune oophorite inducement. The effect is characterised by the presence of lymphocytic infiltration in the ovary. Superficial forms (first and second degree) were associated with the presence of infiltrates, predominantly localised in the interstitial region of the ovary and involved atresic follicles. A serious form was associated with a diffuse infiltration involving the developing follicles, with the presence of giant cells as well as with granuloma formation or atrophy.

ZP3 present on the primordial follicles and in the granulosa cells could represent the cause which generates ovarian pathology following immunisation with ZP3 (Groothenhuis *et al.* 1966). Thus, the interruption of feedback signals

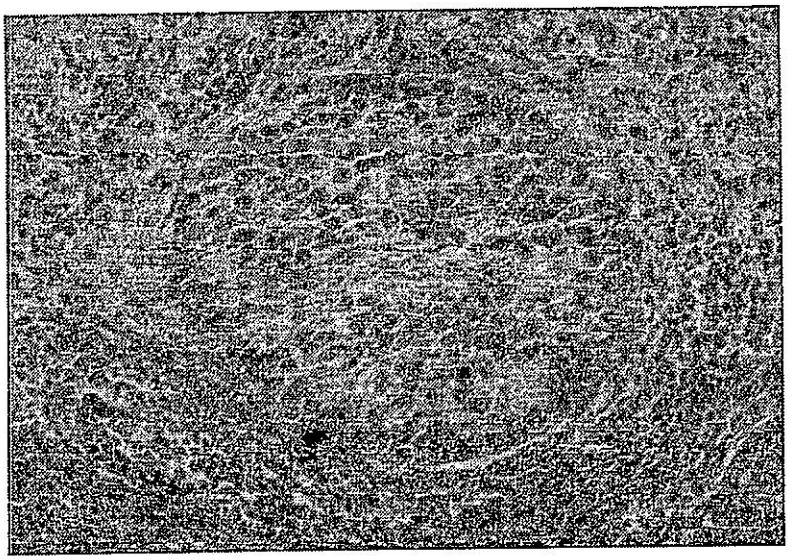

Figure 4. Oocyte loss from the ovary of an immunised female from group D

from the recruited follicles towards primordial follicles might induce fast recruiting of the whole pool of primordial follicles in response also, the antibodies and conjugated complement system may create a toxic micro medium for sensitive primordial follicles.

Using chimerical peptide CP2 as well as its modified alternative CP1, having the same 13 unit polymeric peptide derived from mouse ZP3 ($mZP3^{330-342}$), Yahuan *et al.* (1995) managed to induce anti ZP antibodies and afferent reversible infertility, without inducing ovarian pathology.

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

CONCLUSIONS

Immunisation of bitches with sow oocytes with adjacent *zona pellucida* influences the meiotic competence of their oocytes.

The percentage of cumulus oocyte complexes which are no longer able to gain meiotic competence as well as the degree to which these complexes resume and undergo meiosis decreases with increasing number of boosters.

From the histological point of view, depending on the number of boosters, different degrees of oocyte number decrease as well as serious effects on follicular growth and development were observed. After the second booster (third immunisation) oocyte containing follicles with normal appearance, capable to undergo upper levels of development, were no longer detected.

Further study are necessary to confirm if shorter intervals between immunisations with porcine ZP can be more effective and potentially lead to the development of a contraceptive vaccine for dogs.

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UTICAJ IMUNIZACIJE ZONOM PELUCIDOM KRMAČA NA JAJNIKE KUJA

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SADRŽAJ

Postoje brojni eksperimentalni dokazi o efektima ksenoimunizacije antigenima zone pelucide na reproduktivne funkcije ženki sisara. U ovom radu su izneti rezultati ispitivanja efekata imunizacije kuja zonom pelucidom krmača na sposobnost oocita da završe mejotičku deobu kao i promena u histološkoj građi jajnika. Sa povećanjem broja imunizacija smanjivala se sposobnost oocita kuja da uđu u proces mejotičke deobe a osim toga, histološke alteracije jajnika su bile izraženije. Ove promene su se pre svega manifestovale gubitkom folikula i oocita.