Immunocontrol in dogs

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Abstract

Population control in dogs and cats is an important goal for many groups. Control measures over the years has included surgery, hormonal therapy and more recently immunological control. The current presentation discusses dog population control with an emphasis on immunologic control. Specifically, vaccination with purified zona pellucida ZP glycoproteins leads initially to immunocontraception and then to the profound and irreversible changes of immunosterilization. The preliminary studies are extremely encouraging on developing a vaccine for lasting canine population control. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Mammalian fertilization involves a series of intricate events. One of the most crucial steps that must take place prior to fertilization is the ovulation of an ovum with an intact and mature zona pellucida (ZP). The ZP glycoproteins are highly glycosylated and these carbohydrates moiety is necessary for the process of fertilization. Generally, the sperm cell must penetrate the cumulus matrix, recognize and bind to the specific surface zona carbohydrates then penetrate the ZP.

As the target of sperm binding the ZP plays a pivotal role in fertilization. It has therefore become a desirable target for immunocontraception. Mechanistically, disruption or blocking of the carbohydrate and/or protein receptor sites or chemical modification of the ZP generally will result in immunocontraception. Specifically, the production

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of anti-ZP antibodies and their binding to the ZP results in immunocontraception. However, some species need irreversible population control and in these instances immunosterilization is the primary goal. Immunosterilization results after vaccination with a specific target protein that leads to a massive T-cell reaction. If the reaction is substantial enough the reproductive integrity of the ovary will be destroyed and the female is sterilized by an immune reaction. This immunosterilization is the best technology for dog and cat population control. In order to understand the pathophysiology of immunocontraception and immunosterilization, the biology of the canine ovary, the oocyte and zona pellucida is reviewed. This provides the background for the discussion of the mechanism of canine immunocontraception and immunosterilization.

2. Structure of the carnivore ovary

Morphologically, the canine and feline ovaries appear similar to those of many other domestic species in that it is composed of an outer cortical region and an inner medulla. The internal region or medulla is characterized by the presence of nerves, large coiled blood vessels and lymph vessels embedded in loose connective tissue that contains strands of smooth muscle. Retie ovarii, which are networks of irregular channels lined by cuboidal epithelium or solid cellular cords, are prominent in the medulla of carnivores. These, when appositioned to an oocyte, are thought to differentiate into follicular cells.

The peripheral or cortical region of the ovary is occupied by follicles in various stages of development or corpora lutea embedded in a loose connective tissue stroma. The corpora lutea of carnivores and rodents, contain cords of polyhedral interstitial endocrine cells. Additionally, the ovary of the bitch contains narrow, cuboidal epithelium-lined channels called cortical tubules, which are continuous with the layer of cuboidal epithelium that overlies the thick connective tissue layer, the tunica albuginea of the ovary (Preidkalns and Leiser, 1998).

2.1. Classification, structure and development of follicles and oocytes

Oogenesis, the production of oocytes from primordial germ cells, begins prenatally by mitotic proliferation of the internal epithelial cell masses in the ovarian cortex. The internal epithelial cell masses are considered to arise from interaction of several cell types. These include the cortical stroma, ovarian surface epithelium or the rete ovarii and primordial germ cells which embryologically arrive in the gonadal ridge from the entoderm of the yolk sac (Noden and DeLahunta, 1985). Unlike other species, oogenesis in the dog may occur for about 2 months after birth since proliferating cells, resembling germ cells, have been observed in small clusters throughout the ovarian cortex (McDougall et al., 1997).

Follicles are classified variously, based on size, numbers of layers of follicle cells surrounding the oocyte, the appearance of the zona pellucida and the appearance of the follicular antrum (Tesoriere, 1981). In its most basic form, an ovarian follicle is composed of an oocyte enclosed by specialized epithelial cells. As the follicle undergoes
further differentiation, an additional layer of specialized stromal cells envelops the oocyte with subsequent differentiation of a fluid-filled cavity or follicular antrum within the stroma.

2.2. Primordial follicles

The earliest recognizable form of the female gamete, the primordial follicle, is seen from about 3 weeks after birth and occurs in clusters in the cortex of the ovary (McDougall et al., 1997). The follicles are small and contain a primary oocyte surrounded by a single layer of squamous epithelial cells (follicular cells) that rest on a basement membrane. The oocyte contains a large dictyate nucleus (arrested in diplotene stage of first meiotic division) with a distinct nucleolus. The ooplasm contains large rounded mitochondria, smooth endoplasmic reticulum and small Golgi bodies (Tesoriero, 1981). Gap junctional contacts are also present between the microvilli of follicle cells and the oocyte membrane. Desmosomes anchor the follicle cells to each other (Anderson and Albertini, 1976).

2.3. Primary follicles

The next stage in the development of the solid growing follicles is the primary or preantral or unilaminar follicle. The follicle is characterized by an increase in size and enclosure of the oocyte by a layer of simple cuboidal epithelial follicular cells (Preidkalns and Leiser, 1998). The zona pellucida is immunologically detectable, although it may not be completely circumferential (Fayrer-Hosken, 1999 personal communication).

2.4. Secondary follicle

The progression of development to the secondary follicle is characterized by significant changes in morphology. Specifically the enclosure of the oocyte by a stratified epithelium of polyhedral follicular cells termed granulosa cells. These morphological changes include dilatation and budding of the outer lamellae of the nuclear membrane as well as increases in mitochondria, endoplasmic reticulum, Golgi bodies and granularity of the matrix of the ooplasm. All of these organelles are consistent with increased synthetic activity within the cell. A unique feature of the dog, cat and porcine ova at this stage is the appearance of large amounts of lipid yolk material. The yolk material first appears in association with a single centriole and is directly associated with lamellae of the smooth endoplasmic reticulum. At this stage, the zona pellucida continues to form as aggregates of material that coalesce to form a continuous layer between the oocyte and the adjacent granulosa cells. Focal areas of contact are maintained through the zona pellucida by cytoplasmic extensions of the granulosa cells to the oocyte (Tesoriero, 1981; Preidkalns and Leiser, 1998).
2.5. Tertiary follicles

As oocyte development progresses to the tertiary follicle, the nature of the follicle changes to an antral or vesicular body which characterizes the Graafian follicle. At this stage, the oocyte is enclosed by a layer of stratified epithelium of granulosa cells, which are delimited by a basement membrane. On the exterior of this membrane a multilaminar layer of stromal cells called thecal cells develops. The thecal cells differentiate into an external layer which is essentially supportive, and an inner layer which is vascular. As the follicle develops, small fluid filled clefts develop among the granulosa cells and these later coalesce to form the large antrum which fills with fluid, the liquor folliculi. As the antrum develops, the oocyte becomes enclosed in a collection of granulosa cells termed the cumulus oophorus that later become columnar and radially disposed, forming the corona radiata. The corona radiata is believed to supply nutrients to the oocyte (Preidkalns and Leiser, 1998). Within the oocyte cytoplasm, adjacent to the granulosa cell-contact areas, are large Golgi, numerous mitochondria and dense granular vesicles containing cortical granules. Dense cortical granule-like vesicles are also noted within lamellar spaces and are thought to be associated with a mechanism whereby material is added to the yolk bodies. As the oocyte continues to grow, the amount and size of the lipid accumulation in vesicular bodies increase but do not coalesce (Tesoriero, 1981).

3. Ovulation

While monovular follicles are the most prominent feature of the ovary until just before first estrus, several polyovular follicles, containing two or more ova, are present in the ovary at all ages (McDougall et al., 1997). Additionally, the increases in the total number of monovular follicles and in oocytes found in monovular follicles at first estrus may be indicative of the establishment of a population of follicles and oocytes for subsequent ovulations at mature ages (McDougall et al., 1997). Ovulation is characterized by rupture of several mature follicles in the dog and results in the development of several corpora lutea in the cortex of the ovary. Subsequent to increased follicular blood capillary pressure and permeability associated with estrus there is an increased accumulation of liquor folliculi, which leads to follicular rupture. As the follicular wall swells it becomes thinner and more transparent at the stigma, which is the site of future rupture of the follicle (Preidkalns and Leiser, 1998). Rupture is thought to be related to the release of collagenase as a result of prostaglandin activity mediated by the release of luteinizing hormone (Preidkalns and Leiser, 1998). At ovulation, the follicle collapses and blood fills the antrum resulting in the formation of a corpus hemorrhagicum. After ovulation, the stratum granulosum becomes highly vascularized via capillaries from the theca interna. The granulosa cells undergo hypertrophy and hyperplasia with accumulation of lipid (yellow pigment) which is characteristic of luteinization and formation of the corpus luteum. Luteal regression is characterized by condensation of lutein pigment, fibrosis and resorption of most of the corpus luteum resulting in a connective-tissue scar remnant termed a corpus albicans (Preidkalns and Leiser, 1998).
The unique feature of ovulation in the dog, in contrast to other mammalian species, is the release of an immature oocyte containing a germinal vesicle, which has to mature within the oviduct (Tsutsui, 1989). The immature germinal vesicle is characterized by a vesicular nucleus with a distinctive nucleolus surrounded by fine filaments (Hewitt and England, 1998). This immature oocyte requires at least 48 h to complete its meiotic maturation (Tsutsui, 1989; Hewitt and England, 1998) and the ovulated canine oocyte can remain fertile, in vivo, for up to 108 h (Tsutsui, 1989). Accordingly, to ensure fertilization of mature oocytes, spermatozoa may remain viable for as long as 268 h in the estrous female genital tract after mating (Doak et al., 1967). Further, in vitro studies have demonstrated that canine sperm can penetrate homologous immature oocytes (Mahi and Yanagimachi, 1976), which suggests that in vivo sperm penetration may occur in the oviduct prior to completion of oocyte maturation. In contrast to previous reports, histological examination of ovarian oocytes in situ has demonstrated the presence of germinal vesicle breakdown nuclear material in some oocytes. This suggests follicular maturation may occur to a limited extent within the ovary; however, these may simply represent a transitory state or material obtained from atretic follicles (Hewitt and England, 1998).

The ZP is an extracellular glycoprotein matrix which surrounds the canine oocyte and serves to protect the underlying ooplasm and contains specific receptors for spermatozoal binding. The canine zona pellucida, in common with other mammals, consists of three glycoproteins, ZP1, ZP2 and ZP3. The sequences of these proteins have been reported (Harris et al., 1994) and they have significant homology with the ZP glycoproteins of other species. Specifically, there is homology with the ZP proteins of the pig. But of greatest interest to immunocontrol of the dog, there are significant distinctive differences in the glycosylation of the dog ZP glycoproteins (Barber et al., 1999) compared to the pig glycoproteins.

4. Immunocontraception

Interest in the ZP as a potential target for mammalian immunocontraceptive and immunosterilant vaccines has arisen because of its importance in fertilization, its unique expression in oocytes, and its strong immunogenicity. If the ZP is masked or structurally altered, fertilization will not occur and one would have an immunocontraceptive vaccine. Thus, much research has focused on the generation of anti-ZP antibodies to specific epitopes that inhibit fertilization without altering ovarian function. Specifically, there has been the administration of porcine zona pellucida (pZP) glycoproteins, which has resulted in immunocontraception in many species of mammals (Kirkpatrick et al., 1996, 1997; Fayrer-Hosken et al., 1997a,b). Immunocontraception can be defined as the ability to use a reproductive protein to produce a humoral immune response that leads to the animals immunocontraception for a defined time period. At the end of this period the amount of circulating antibodies, IgG, decreases and the animal becomes fertile.

In theory, the general mechanism of immunocontraception is simple and we have hypothesized that antigens, which in this case are ZP glycoproteins, are presented in a manner that results in the production of anti-ZP IgG antibodies. These antibodies then
block fertilization primarily at the site of sperm–zona interaction. In reality, the underlying mechanism of immunocontraception is actually quite complex. Immunocontraception probably interferes with one or several mechanisms that cause a cascade of biochemical events leading to infertility.

It has been shown (Henderson et al., 1988) that anti-pZP antibodies bind to sperm receptor sites of the host zona and ultimately inhibit spermatozoal binding. These findings are further supported by earlier studies (Sacco et al., 1989) in which pretreatment of porcine oocytes with antibodies to pZP3α (primary sperm receptor) blocked sperm binding, while pretreatment of oocytes with antibodies specific to pZP3β (secondary sperm receptor) did not inhibit sperm binding. It has also been suggested (East et al., 1985; Mahi-Brown et al., 1985) that anti-pZP antibodies do not simply inhibit sperm binding to the oocyte, but in fact result in an inhibition of sperm penetration. In the study by East (East et al., 1985) using a mouse model, monoclonal antibodies to both the primary and secondary sperm receptors in the oocyte (ZP3 and ZP2, respectively) did not interfere with sperm binding, but did reduce sperm penetration. The study by Mahi-Brown (Mahi-Brown et al., 1985) in that same year reported similar findings in the bitch in that inhibition of sperm penetration was the primary finding that explained the immunocontraception in these animals. In fact, these authors suggested that perhaps sperm penetration was inhibited via an inhibition of the acrosome reaction of the spermatozoa. From this it could be argued that the immunocontraception may be a consequence of altered sperm–zona attachment or a modification of the sperm’s ability to penetrate the zona pellucida, or across species a combination of both precepts.

Another potential mechanism of immunocontraception may involve a change in structure of the zona pellucida itself. In most species, after sperm bind to and penetrate the oocyte, a cascade of biochemical events results in the release of peripheral cortical granules, the cortical reaction. The release of the cortical contents into the perivitelline space induces zona hardening and ultimately prevents penetration by additional spermatozoa (zona block). It has been proposed (Ducibella, 1996) that binding of anti-ZP antibodies to the oocyte cause a premature activation of the oocyte. Thus the potential oocyte activation leads to the cortical reaction which leads to zona hardening and the spermatozoa are incapable of binding or fertilizing the egg.

The vaccination of dogs with pZP initially causes a rise in serum IgG levels. These levels are enough to block fertilization and immunocontracept the bitch. Bitches that have been vaccinated with our vaccine have been immunocontracepted for several months. However, the vaccinated dogs with significantly elevated serum IgG levels also show marked ovarian pathology. This pathology is the preliminary evidence of impending permanent ovarian damage and eventual immunosterilization.

5. Immunosterilization

Immunosterilization involves the ability to use pZP glycoproteins to produce an immune response that leads to the animals sterilization by destroying oocyte/granulosa cell complexes or causing ovarian follicular atrophy. However, the pathophysiology of immunosterilization is not completely elucidated for all affected species. The pathophys-
iology of mammalian ovarian disease is multifactorial. There are clearly several distinct clinical presentations, ranging from premature ovarian atrophy to polycystic ovarian disease to autoimmune oophoritis. The pathogenesis of the naturally occurring disease involves the humoral and cell mediated immune responses. However, the pathophysiology of immutable, induced ovarian disease is not as clear. Also the immunological and pathological differences among affected species is paradoxical.

The genetic predisposition of an animal to the disease mediates the severity of the immune response (Tung et al., 1997). Thus, some species or certain population groups within a species might be the most susceptible to ovarian pathology. In all affected animals, the final disease is a result of antigen presentation and the sequence and severity of the immune response.

Antigen presentation is the most important facet of current research. Most researchers are trying to define a synthetic, immunocontraceptive molecule. This would be a small epitope that would not cause any ovarian pathology. In our research, we are investigating a safe, yet permanent immunosterilization and therefore aggressive antigen presentation is essential. The molecule itself must be antigenic, so we use a heterologous molecule. Also, the presented antigen is enhanced by an adjuvant. For our studies the pZP molecules provide the necessary prerequisite features for an immunosterilant vaccine.

For the vaccinated animals, the individual immune response is also pivotal to ensure an immunosterilant effect. In several species, female subjects immunized with heterologous ZP developed ovarian pathology, but the mechanism of the ovarian diseases remained undefined (Skinner et al., 1984; Mahi-Brown et al., 1985; Henderson et al., 1988). However, over time excellent studies by Tung et al. (1997) showed that ovarian pathology was a result of (a) immunoglobulin priming and T-cell response, and (b) B-cell mediated responses. In a study on murine ZP peptide, outbred CD1 mice immunized with a 15-mer murine ZP peptide developed reversible infertility (Millar et al., 1989). However, this ZP peptide was subsequently shown to possess T-cell epitopes and to induce oophoritis, an autoimmune disease induced by CD4+ T cells (Rhim et al., 1992; Luo et al., 1993).

An additional problem regarding ZP vaccine development is the genetic polymorphism of autoimmune responses to the ZP glycoproteins. Previous reports had shown the use of a chimeric peptide with a promiscuous T-cell epitope as an effective vaccine formulation (Greenstein et al., 1992; Partidos et al., 1992; Su and Caldwell, 1992; Lopez et al., 1994). Tung et al. (1997) modified this strategy by altering the ZP epitope so that the T-cell epitope of ZP was completely eliminated. This produced an antibody to ZP for immunocontraception induction, yet insufficient for induction of autoimmune oophoritis. However, for an immunosterilant vaccine retention of the T-cell epitopes has been important to produce adequate ovarian pathology. Pathologically, the cytotoxic T-cells lead to autoimmune disease of the ovary and secretion of cytokines by the immune cells probably have an adverse effect on ovarian steroidogenesis. Therefore, the immunosterilant vaccine has an effect on the female reproductive system by affecting the endocrine and the immune systems.

For our studies enzyme-linked immunosorbent assays (ELISAs) revealed that the serum antibody levels of vaccinated, immunosterilized dogs were significantly \( p < 0.05 \)
elevated when compared to controls vaccinated with a placebo. Antibody levels rose slightly after the initial vaccination and then significantly \((p < 0.05)\) after the first and second boosters. These data clearly show that there is a significant immune response to the pZP vaccine and adjuvant. Furthermore, normal dog ovaries have distinct oocytes with a zona pellucida, especially in primary, secondary and tertiary follicles. Immunohistochemical studies of these normal ovaries indicated that there was marked staining of the zona pellucida in these follicles with anti-pZP antibodies. This can be compared and contrasted to ovaries of vaccinated immunosterilized dogs. The ovaries of immunosterilized dogs revealed histologically that the integrity of all follicles has been breached. All follicles have been significantly invaded by leucocytes and specifically neutrophils, lymphocytes and macrophages. With immunohistochemical studies it was clear that there was an immunologic response at the site of the zona pellucida of the oocytes, and in fact no immunodetectable zona pellucida glycoprotein material remained. It is evident from these studies that the oocyte–granulosa cell complexes in the canine ovary have been disrupted.

6. Conclusion

In conclusion, recent reports have indicated that immunocontraception via zona pellucida immunization is a highly promising avenue for providing alternative means of contraception. We present preliminary data that permanent immunosterilization can also be achieved in dogs. Immunosterilization would be an effective and practical technology for controlling dog populations throughout the world.

References


