

ZONA PELLUCIDA: AN EXTRACELLULAR MATRIX WITH APPLICATIONS IN THE STUDY OF IMMUNOCONTRACEPTION IN DOMESTIC CARNIVORES

Zona Pelúcida: Una Matriz Extracelular con Aplicaciones en Anticoncepción de Carnívoros Domésticos

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ABSTRACT

Zona pellucida (ZP) is an extracellular matrix surrounding the mammalian oocyte. In most mammalian species this matrix consists of three families of glycoproteins likely to suffer several postraductional modifications to acquire different immunological and biochemical properties. The ZP are involved in the initial recognition and binding events of sperm to oocyte investments. Because of its major role in the fertilization process (interaction with sperm, induction of acrosome reaction, control of polyspermy) the ZP has been used as an antigen to induce immunological response in order to produce female infertility, a relevant issue in domestic carnivores. Depending on the predominant immunological response (humoral or cellular) the infertility can be reversible (Immunocontraception) or irreversible (immunosterilization), respectively. Currently the goal is to develop recombinant ZP antigen with immunodominant epitopes in order to produce predictable and safely response.

Key words: Zona pellucida, vaccines, anticonception, feline, canine.

RESUMEN

La zona pelúcida (ZP) es una matriz extracelular que rodea al ovocito en los mamíferos y está constituida por tres familias de glicoproteínas en la mayoría de ellos, que difieren en sus propiedades inmunológicas y bioquímicas. La ZP participa en los eventos de reconocimiento y unión del espermatozoide a las

envolturas del ovocito, siendo una de sus principales funciones la participación en el proceso de fecundación (interacción con el espermatozoide, inducción de la reacción acrosómica, control de la poliespermia). La ZP ha sido utilizada como antígeno para inducir una respuesta inmune tendiente a disminuir la fertilidad de las hembras, condición que adquiere especial relevancia en el caso de los carnívoros. Se ha observado que dependiendo del tipo de respuesta inmune predominante -humoral o celular- el efecto en la fertilidad puede ser reversible (inmunoanticoncepción) o irreversible (inmunoesterilización) respectivamente. En la actualidad el propósito es el desarrollo de antígenos de ZP recombinantes, con epítopes inmunodominantes, con el objeto de inducir una respuesta inmune predecible y segura.

Palabras clave: Zona pelúcida, vacunas, anticoncepción, felino, canino.

INTRODUCTION

The research on immunological techniques to control the reproductive activity has increased during the last decade, as an alternative to develop new compounds to promote a better management of the fertility in different species [7, 51].

The regulation of the fertility in domestic animals, especially carnivores, is an important issue due to the sanitary implications (human and animal health) and ecological due to the increase of the homeless animals population, in both rural and urban areas. The demographic control policies in relation to cats and dogs are designed to approach two aspects: a) the elimination of the animals using humanitarian methods which normally generate controversy from a judicial – ethical perspective and b) the fertility control through surgical and non

surgical methods, which can be variable in terms of effectivity, cost and risks [42]. The most popular birth control methods in bitches and cats has been the surgical removal of the gonads; this procedure however, is very expensive when applied to massive population control. A promising alternative for fertility control in massive programs could be immunoanticonception using gamete antigens as immunogens, which can alter the fertilization and/or oocyte production processes due to their capacity to evoke an immune response [6].

One of the crucial stages in reproduction is the interaction with and penetration of the spermatozoa into the zona pellucida. Spermatozoa must recognize and bind to specific carbohydrates on the zona pellucida, in order to penetrate it and undergo the fusion of the spermatozoa with the oocyte plasma membrane to start the process of fertilization [8]. Antibodies binding either the spermatozoa or the oocyte or their layers would block some stages of the gamete interaction thus preventing fertilization.

At present, the development of anticonceptive vaccines for dogs and cats is a worldwide great interest mainly due to both the need to regulate the homeless animal populations and consequently to decrease the cost involved in the capture and euthanasia, and the high sanitary risk for the human population involved [33, 34].

ZONA PELLUCIDA

Zona pellucida (ZP) corresponds to an extracellular porous and trabecular matrix of a fibrogranular structure surrounding the mammals oocyte with specific functions during fertilization (8) and the early stages of embryonic development [55]. Interaction with the sperm, induction of the acrosome reaction and prevention of polyspermy during fertilization are amongst the functions of ZP [58]. During the embryonic development ZP prevents disaggregation of the noncompacted blastomeres and the premature attachment to the oviductal and endometrial surface. Additionally, it protects the embryo against toxins and xenobiotics, as well as bacteria, viruses and phagocytes and facilitates the signal transmission between the embryo and the uterus [15].

The single most distinctive morphological feature of the ZP of different mammalian species is that of relative size. This matrix is more pronounced than other extracellular matrices and varies in size from 5 µm in the mouse (*Mus musculus*), 13-16 µm in the human and the pig to 27 µm in the cow. The porous nature of this matrix allows penetration of large molecules such as immunoglobulins and ferritin but is reported to be impermeable to smaller molecules such as heparin. Therefore, the ability of molecules to pass through the ZP matrix apparently does not depend on the relative size of the molecule but on other biochemical and physicochemical properties [38, 40].

In mammals, ZP is mainly formed by sulphated glycoproteins which represent about 95% of the total mass of the extracellular matrix. These glycoproteins are synthesized by the oo-

cyte and/or the granulosa cells of the ovarian follicles at every stage of follicular development [40].

The ZP glycoproteins of most mammalian species have been classified in three families according to their electrophoretic migration patterns. Initially, the studies were carried out using mouse ZP and the following proteins were observed: ZP1 (180-200 kDa), ZP2 (120-140 kDa) and ZP3 (83 kDa) [4].

Similar structural domains have been identified in each protein family. The common domains for the three protein families are; a ZP module or hydrophobic domain containing 260 aminoacids and eight cysteine residues which probably determine the three-dimensional structure, a transmembrane hydrophobic domain located in the carboxy terminal, and a proteolytic processing signal located anterior to the transmembrane domain, showing similar glycosylation consensus in asparagine [38].

From the structural point of view, each family consists of proteins with the same polypeptidic skeleton. In general, it is accepted that the structure of ZP1, ZP2 and ZP3 glycoprotein families is highly preserved amongst the studied species, with a degree of homology fluctuating between 50% to 98% [40].

It is worth noting that although the polypeptidic structure of such proteins is highly preserved in nature, there are important postraductional modifications including glycosylation and sulphating that could be responsible for the structural and functional heterogeneity of the proteins [47].

It has been reported that the principal molecules related with the oocyte – spermatozoa interaction are the carbohydrate residues present in ZP proteins [59]. The latter aspect has complicated the classification of the ZP protein families when comparing different animal species. Only in the last decade and through the study of complementary DNA (cDNA) it has been possible to identify the genes and the families of the genes encoding for such proteins [13].

At present, an accepted nomenclature for the classification of ZP proteins takes into consideration structural aspects specially the molecular weight derived from the cDNA sequences, in comparison with ZP1 (68 kDa), ZP2 (80 kDa) and ZP3 (46 kDa) families in mouse [29, 45] (TABLE I).

It is known in the female cat that ZP proteins have a sequential pattern of synthesis in the ovary with the early expression of ZPB in primary follicles whilst, ZPA and ZPC are expressed later in secondary follicles [18]. In mice the oocyte it-

TABLE I
CLASSIFICATION OF ZONA PELLUCIDA (ZP) PROTEINS
[13, 29, 44]

Mouse	Human	Porcine	Rabbit	Feline	Canine
ZP1	ZPB	ZP3	R55	ZPB	ZP1
ZP2	ZP2	ZP2	R75	ZPA	ZP2
ZP3	ZP3	ZP3	ZPC	ZPC	ZP3

self is capable of synthesizing all ZP proteins [39]. In other species such as the domestic bitch the granulosa cells also contribute to the synthesis of these proteins [36, 49]. In the female cats however, it has been established that during all stages of follicular development the ZP synthesis is carried out by the granulosa cells with the participation of the oocyte [20].

The functions of zona pellucida would be common to the ZP proteins of different animal species thus; a strategy to modify the fertilization process could be to consider the immunogenic characteristics of the numerous antigenic determinants, such as carbohydrates, proteins and conformational epitopes to induce an immune response that provides an anticonceptive effect [18, 47].

The crossed immunoreactivity shown by ZP glycoproteins amongst several species could explain the presence of homologous sequences in the polypeptidic chain and consequently the heterologous immunization [41].

SPERM - EGG INTERACTION

The principal molecules involve in the sperm-egg interaction are carbohydrates residues present in ZP proteins [59]. It has been suggested in the mouse that sperm receptor activity on the egg is associated with O-linked carbohydrates moieties on one specific zona pellucida glycoprotein, ZP3 [53, 56]. This binding property is associated to the carbohydrate component (O-linked oligosaccharides) as it was not affected when treating ZP3 with pronase or endo-N-acetylglucosaminidase F [25]. The polypeptidic chain however, could be responsible for the induction of the acrosomic reaction [54].

It has also been shown in the mouse that following the acrosome reaction the ZP3 receptor is released and the sperm could bind to the zone through a second receptor (a ZP2 receptor) located in the acrosomic internal membrane and it is likely to be proacrosine (8). It is suggested an alternate steps mechanism of binding and release where the sperm is binding and releasing through the controlled digestion of the zona pellucida by the acrosine [22, 35]. Thus, the oligosaccharides present in ZP2 would be exposed and keeping a strong association with the sperm.

Following the penetration of zona pellucida by the sperm there is the activation of the oocyte with biochemical changes that will produce, among other changes, the reaction of the zona with the subsequent release of the cortical granule content into the perivitelline space thus, preventing polyspermy [58]. The early activation of oocyte through the anti-ZP antibodies binding could make the oocyte refractory to the spermatozoid and as a consequence become another immunological mechanism capable of altering fertilization [10].

THE ZONA PELLUCIDA AS IMMUNOGEN

At present, ZP proteins due to their antigenic capacity appears as the principal alternative to produce anticoncep-

tive vaccines [5, 7, 17, 29]. In addition, the ZP antigens are specific for the reproductive system and they do not reach the blood stream; thus, it is not likely to observe the development of immune complexes and alterations in other tissues [2]. On the other hand however, it has been observed that the induced immune response could be variable and dependant on the immunogenic structure used as well as the immunized species [32, 47].

Due to the fact that ZP glycoproteins are weak alloantigens, the immunization of the animals has been done using zona from other species. Thus, the antibodies against glycoproteins from pig zona pellucida show cross reaction with glycoproteins from zonas of other species [1, 10, 23]. This has induced the isolation and purification of porcine zona pellucida proteins and the use of them as vaccines in different wild and domestic mammal species [7] thus, supporting the idea that the induced antibodies adhere to the ZP of the oocytes of the treated animals and block fertilization by preventing the binding and penetration of spermatozoa [10, 23].

Considering the antigenic characteristics, the use of pZP as contraceptive vaccine in carnivores is controversial. In that sense for example, it has been described that cat and pig ZP express and share a very small number of antigenic determinants, which means that the use of pZP vaccine in cats is still unclear [19]. However, it has also been observed that canine and feline ZP are recognized by antibodies against pZP, this suggest similar antigenic properties [1]. The latter has been corroborated by immunohistochemistry studies showing that the induced antibodies in immunized female cats with a vaccine based on pZP (Spay VacTM) recognize the porcine zona pellucida but not the feline zona pellucida [11].

On the other hand, it has been pointed out that in domestic carnivores one of the main difficulties of the immunization using pZP purified proteins is the irregularity of the results in terms of size and duration of the immune response, variability in the anticonceptive effect and the presence of pathological and functional alterations in the ovaries [16, 27, 28, 31, 32]. The latter could be acceptable and in some cases recommended for the development of permanent immunocontraceptives in some animal populations, but undesirable to control the reproduction of animals of commercial value. It is important therefore, to determine which proteins or epitopes from ZP do not alter the development of the ovarian follicle.

By using antibodies anti-pZP derived from rabbit it was observed that they were not effective in inhibiting the homologous sperm/oocyte binding neither the *in vitro* fertilization in felines [19]. It has recently been reported little anticonceptive effect in female cats using a commercial vaccine based on pZP (Spay VacTM) thus, confirming the scarce cross reaction of feline antibodies against pZP with own fZP antigens [11].

In relation to the control of the dogs and cats populations, the immunoanticonception has been defined as the possibility to use a protein of reproductive origin capable of evok-

ing a humoral immune response, to produce an anticonceptive effect for a well defined period of time and at the end of such a period of time, the amount of circulating antibodies will decrease and the female will recover her normal fertility [10].

Other authors reported that the stimulation of the immune system with proteins or peptides containing dominant epitopes to stimulate the cellular immunity and therefore capable of generating inflammatory and irreversible modifications in the ovary, could be another alternative for the fertility control in those species, defining this last option as immunosterilization [5, 26]. Similarly, the infertility induced by the ZP antigens inoculation could be the result of two actions: first, the blocking of ZP spermatic receptors (humoral immune response) and second, the destruction of the primordial ovarian follicles by cytotoxic T cells (cellular immune response) [7].

Thus, the active immunization using ZP antigens can produce infertility that could be reversible or irreversible depending on the type of dominant epitope. Immunodominant epitopes by B cell will induce a reversible humoral response and immunodominant epitopes for T cells could generate autoimmune complications producing the loss of intraovarian oocytes and permanent ovarian dysfunction [5, 37].

The immune system in domestic carnivores is capable of generating a humoral and cellular immune response. The serum is the fluid containing the highest concentration of immunoglobulins and Immunoglobulin G (IgG) is the most abundant [3]. The follicular fluid shows IgG that transudates from serum therefore, in actively immunized animals with ZP antigens, the anti-ZP IgG could cover the preovulatory oocyte and inhibit the binding of the spermatozoid to ZP [7]. In addition, in the case of the cellular response it is recognized that felines have two important subpopulations of T lymphocytes, CD⁴⁺ and CD⁸⁺ corresponding to 25% and 15% of circulating lymphocytes, respectively [57]. It has been described in rats that T cells could actively participate in the mechanism of induction of the autoimmune ovarian disease characterized by inflammation, loss of functional tissue and atrophy [50].

It has been postulated that one alternative to overcome some of the inconveniences of immunization with pZP is the use of vaccines obtained using genetic engineering and molecular techniques [9, 43, 46] which will be based on recombinant proteins or peptides, homologous or heterologous containing appropriate epitopes to stimulate a specific immune response [21, 29, 30, 37]. Moreover, the contraceptive potential of ZP synthetic peptides with a LDPEKLTLL sequence that inhibits the binding of human spermatozooids to ZP have been recently described [14]. The effect of the antiidiotype antibodies against ZP antigens have been evaluated showing a fertility decrease in immunized female mice [24].

There is little information about the use of this type of antigens in feline and canine. However, the anticonceptive potential of ZP recombinant proteins produced in different

expressing systems have been described in primates [12, 29, 36] and cervids [30].

Studies using rabbit zona pellucida (rZP) recombinant peptides to immunize primates showed that the peptide corresponding to rZP55 protein (homologous sequence to female cat ZPB) will induce the production of antibodies capable of blocking the homologous spermatozoid/oocyte binding without affecting neither folliculogenesis nor ovarian cyclical activity. On the contrary, the active immunization with the rZP75 protein peptide (homologous sequence to female cat ZPA) produced ovarian dysfunction [52].

Similarly, the immunization of primates from the *Papio anubis* species with a recombinant peptide of homologous sequence to r55ZP obtained in another species (*Macaca radiata*) induced high antibody titers producing a reversible blocking of the fertility without an evident ovarian dysfunction [12].

Recent studies evaluating the homologous immunization in bitches using recombinant dZP2 and dZP3 showed an immune response and fertility alterations associated to the production of anti-ZP3 antibodies which could cause the inhibition of follicular development and degenerative changes in the zona pellucida of the immunized females [43, 48].

Although the results from the heterologous rZP immunization in primates suggest that recombinant peptides could contain immunodominant epitopes, this characteristic is now under evaluation in carnivores and this alternative opens an interesting field of research on the temporal as well permanent control of the fertility in such species.

CONCLUSIONS

The analysis of this review shows that the knowledge of the structural and physiological characteristics of the zona pellucida glycoproteins constitutes a tool to apply in the stimulation of the domestic carnivore immune system with the purpose to evoke an immune response capable of altering or blocking the fertility either by producing antibodies capable of inducing reversible anticonception or the induction of a cellular immune response leading to an ovarian failure with inhibition of folliculogenesis resulting in irreversible anticonception. In addition, the use of recombinant DNA technology provides interesting perspectives for the immunocontraception due to the possibility of specific stimulation of the immune system and to control the fertility in females in a temporal (immunoanticonception) or permanent (immunosterilization) way.

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