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Zona pellucida as a target for immunocontraception

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The zona pellucida (ZP) has generated considerable interest as a target for the immunocontraceptive vaccine, blocking pregnancy at pre-fertilization stage. Antibodies against porcine ZP3 have been shown to inhibit sperm–egg interaction. The immunological cross-reactivity among the various species of ZP glycoproteins, has led to the possibility of heterologous immunization. Recent studies in non-human primates using purified ZP components showed reversible infertility without side effects. More recently, peptide immunogens based on the ZP sequence, have become candidate contraceptive vaccines with the demonstration that the deglycosylated ZP components can block fertility with reduced ovarian dysfunction.

KEEPING in view the present rate of growth, it is projected that the human population will cross 6.3 billion mark by 2000 AD and 10–12 billion by 2050 AD. Moreover, most of this population explosion would be in the developing world, further worsening the living conditions in these countries. This has necessitated the need to develop newer and safer methods of contraception. Vaccines regulating fertility are recent entrants in this field. Immunocontraception entails generating either humoral (antibody) or cell-mediated immune response (CMI) against the molecule(s) having a crucial role in the process of reproduction. An ideal immunocontraceptive vaccine should be (i) effective in preventing conception, (ii) potentially reversible, (iii) available in large quantities at reasonable expense, and (iv) free of any side effects. Such vaccines can act at either pre- or post-fertilization stage. To design immunocontraceptive vaccines aimed at blocking at pre-fertilization stage, antigens pertaining to spermatozoa and egg are being investigated along with other candidates such as gonadotropin releasing hormone (GnRH) and ovine follicle stimulating hormone (oFSH). Amongst the egg antigens, zona pellucida has generated considerable interest[1,2]. The zona pellucida is unique among immunocontraceptive targets by virtue of its location within the female reproductive system, and represents a structure through which sperm must pass to fertilize the ovum. Moreover, the expression of zona pellucida genes is highly specific to the oocyte.

Zona pellucida glycoproteins

The zona pellucida (ZP), an acellular layer surrounding the mammalian oocyte and pre-implantation embryo is formed by the organization of 3–5 families of acidic glycoproteins in most of the species studied[3]. These have been separated by gel electrophoresis or column chromatography. Under non-reducing condition, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of murine ZP reveals three families of glycoproteins ZP1 (200 kDa), ZP2 (120 kDa) and ZP3 (83 kDa)[4]. The human ZP shows ZP1 (90–110 kDa), ZP2 (64–78 kDa) and ZP3 (57–73 kDa)[5]. The porcine ZP shows ZP1 (80–90 kDa) and ZP3 (55 kDa)[6]. The porcine ZP under reducing condition resolves into ZP1 (82 kDa), ZP2 (65 kDa), ZP3 (55 kDa) and ZP4 (21 kDa). Porcine ZP1 revealed immunological cross-

Figure 1. Two-dimensional SDS-PAGE of porcine solubilized isolated zona pellucida (SIZP). Isoelectric focussing is represented in the horizontal dimension and SDS-PAGE in the vertical dimension. a and b represent the acidic and basic ends respectively. The gel was stained with Coomassie blue. Arrow indicates the charge isomers of the 55 kDa ZP3 glycoprotein.

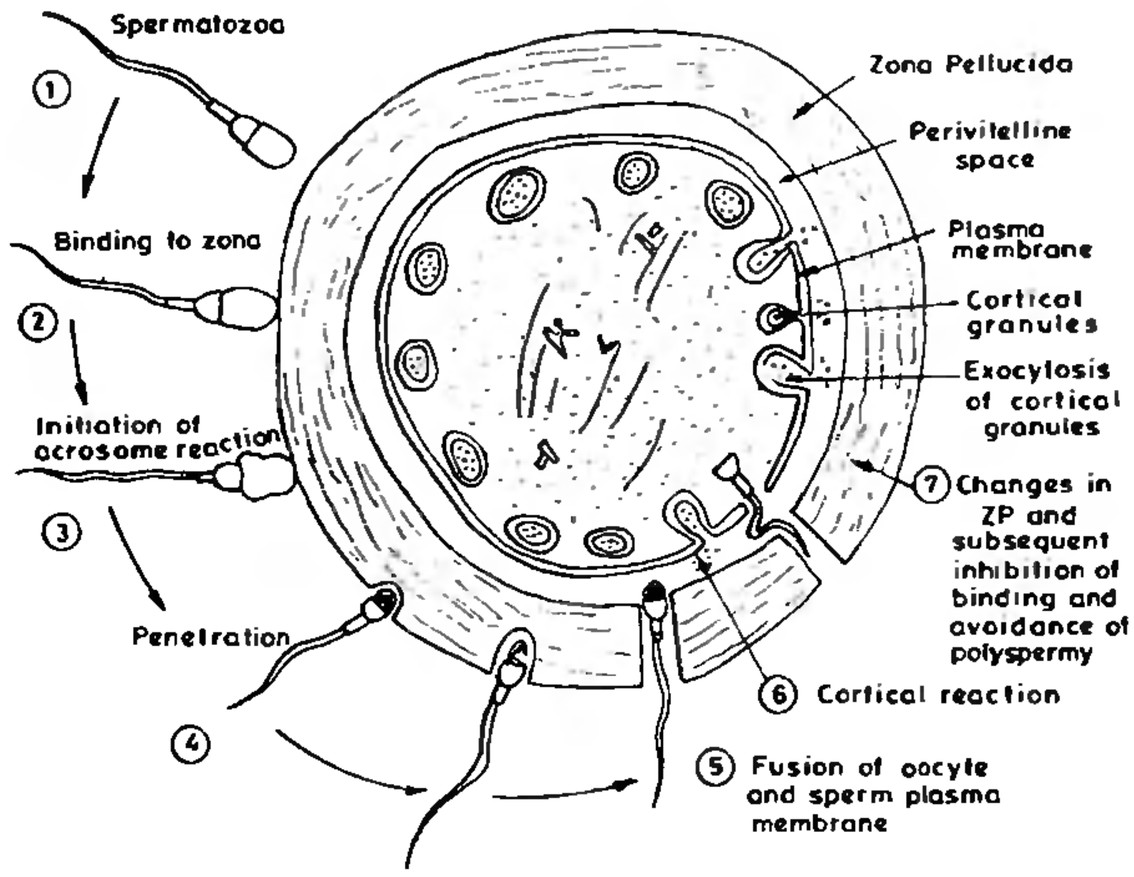

Figure 2. Schematic diagram to show various steps in the process of fertilization. Many sperm can bind to and penetrate the zona but only a single spermatozoa usually fuses with the ovum.

reactivity with ZP2 and ZP4, which do not share reactive epitopes amongst themselves, suggesting thereby that they are derived from reduction of disulphide bonds in ZP1. Porcine ZP3 family consists of a mixture of two biochemically and immunologically distinct glycoproteins, ZP3α (core protein 37 kDa) and ZP3β (core protein 32 kDa). Two-dimensional gel electrophoresis shows that each family exists as several isoelectric species (Figure 1). The ZP3 family consists of 20 charge isomers with apparent PI values of 3.5–6.0. This charge heterogeneity has been attributed to differential glycosylation and not to the polypeptide backbone[7]. The carbohydrate content of ZP glycoproteins is 30–40%. Porcine ZP3α has 4 N-linked and 3 O-linked oligosaccharides (OS) while ZP3β has 4 N-linked and 6 O-linked OS chains. The OS are of branched type, rich in sulphated polylactosamines. Purified ZP3α and ZP3β have been obtained following partial enzymatic deglycosylation by endo-β-galactosidase[6] or by complete chemical deglycosylation[8].

The ZP under electron microscope shows a lattice network with fenestrations showing an inner compact layer with small pores and outer loosely arranged layer with larger pores[9,10]. The mouse ZP consists of 14–15 nm repeat of ZP2 and ZP3 molecules forming long filaments. ZP1 is responsible for cross linking these filaments[11].

Sperm egg interaction: Role of zona pellucida

The interaction between the sperm and the oocyte initiates a complex cascade of events leading to fertilization and formation of the embryo (Figure 2). The acrosome intact capacitated sperm penetrates the layers of cumulus cells and adheres loosely to the outer layer of ZP. Shortly thereafter, the contact becomes more tenacious and the sperm undergoes the acrosome reaction. Once a spermatozoa fertilizes an oocyte, subsequent binding of other sperm is inhibited as a result of the cortical reaction.

These events have been extensively explored in the mouse model[11]. Purification of each ZP glycoprotein to homogeneity and evaluation of its function has revealed that ZP3 is the sperm receptor. Purified ZP3 from ovulated eggs can induce the acrosome reaction in capacitated sperm. However, ZP3 from fertilized eggs cannot induce the acrosome reaction, suggesting modification of the sperm receptor following fertilization, conversely acrosome reacted sperm do not bind to ZP3. The continued binding and penetration has been attributed to the secondary sperm receptor, the ZP2, which binds preferentially to the acrosome reacted sperm. ZP2 also acts as the substrate for the action of the proteolytic enzymes following the cortical reaction. Wassarman has proposed that following fertilization, proteolysis of ZP2 results in the formation of small molecular weight fragments which do not dissociate but remain non-covalently bound[11]. This increase in non-covalent interactions make ZP resistant to proteolytic cleavage and acts as a protective barrier. The exact function of ZP1 is as yet unidentified, but it probably contributes to the structural integrity of the ZP. In porcine gamete interactions, ZP3α is described as the primary ligand for sperm binding[12].

Zona pellucida genes: tissues specific expression

The genes encoding ZP2 and ZP3 are conserved among mammals. Mouse ZP2 and ZP3 genes are single copy genes on chromosomes 7 and 5, respectively. These genes are expressed uniquely in the oocyte and the

products accumulate in the oocyte over a two-week growth phase prior to ovulation. ZP2 and ZP3 genes are co-ordinately expressed and maximum levels of transcripts during the growth phase of the oocyte represent 1% and 0.4% of Poly A(+) RNA respectively. In the latter stages of development of the oocyte, the transcript decreases and by the time the ovum is ovulated, transcripts are present only at around 5% of the peak level. A few transcripts detected in the ovulated egg are shorter by 200 bp, lacking the poly(A) tail, which affects their translation. A comparison of the 300 bp upstream sequence indicates the presence of 5 conserved elements (I, IIA, IIB, III, IV) which are 4–12 bp long, at comparable distance from the transcription initiation site. Studies have demonstrated that the 12 bp element IV is both necessary and sufficient for tissue specific expression of a reporter gene. A protein binding to this sequence has been identified by the gel-retardation assay in extracts of oocyte but not in other tissues[13,14].

The genes for various ZP proteins from different species have been cloned. Mouse ZP3 gene is 8.6 kbp and has 8 exons (92–338 bp) and seven introns (125–2320 bp)[15,16]. It is transcribed into a 1317 nucleotides ZP3 mRNA and also has short 5′ (29 nucleotides) and 3′ (16 nucleotides) untranslated regions. Mouse ZP2 spans 12.1 kbp having 18 exons ranging in size 45–190 bp and 17 introns (81–1490 bp), and is transcribed into a 2201 nucleotide mRNA with short 5′ (30 nucleotides) and 3′ (32 nucleotides) untranslated regions[17]. Human homolog of ZP2 gene is 13 kbp (19 exons), while ZP3 gene is 18.3 kbp (8 exons). The mouse and human ZP2 mRNA encode proteins of 713 and 745 amino acids respectively. The predicted hydropathicity and α-helical content of the two proteins are similar[17]. The mouse and human ZP3 mRNAs both encode proteins of 424 amino acids and have predictable signal peptidase cleavage sites, 22 amino acids from the N-terminus. The mouse protein contains six N-linked glycosylation sites and the human contains four, three of which are conserved between mouse and human. There are over 70 potential O-linked glycosylation sites in mouse ZP3 and 66 in the human[16,18,19]. Both ZP2 and ZP3 have a hydrophobic domain at C-terminus consisting of 23 and 26 amino acids respectively, which may play a role in intracellular trafficking of these secreted proteins or their interactions in the extracellular matrix.

The comparison of human ZP3 to mouse and hamster ZP3 reveals a 60% sequence homology[15,20]. The similarity between mouse and hamster ZP3 is around 81%. Whereas the primary amino acid comparison of human ZP3 to marmoset ZP3 yields a homology of 91% and disparity between these proteins is confined to widely dispersed changes in one or two amino acids, with the exception of the polypeptide chain spanning residues 322–352 in which the homology is reduced to 45% (refs. 15, 21) (Figure 3). Human ZP3 and porcine ZP3 have about 67% homology (Yurewicz, unpublished observation). Despite the conserved amino acid structures of the polypeptide core, differential glycosylation leads to considerable heterogeneity in the molecular mass of the secreted product, giving an apparent molecular weight of 83 kDa for mouse ZP3 and a spectrum of charge isomers ranging from 50 to 60 kDa for human and hamster ZP3 (refs. 22, 23). Taken together, these data suggest that the overall structure of the zona genes and their gene products are relatively conserved among mammals.

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HUMAN      MELSYRLFICLLLWGSTELCYPQPLWLLQGGASHPETSVQPVLVECQEAT 50
MARMOSET   MELSYRLFICLLLWGSTELCYPQPLRLLQGGTSHPETALQPVVVECQEAT 50

HUMAN      LMVMVSKDLFGTGKLIRAADLTLGPEACEPLVSMDTEDVVRFEVGLHECG 100
MARMOSET   LVVTVSKDLFGTRKLIRAVDLTLGPEGCEPLVSTDTEDVVRFEVGLHECG 100

HUMAN      NSMQVTDDALVYSTFLLHDPRPVGNLSIVRTNRAEIPIECRYPRQGNVSS 150
MARMOSET   NSMQVTDDALVYSTFLLHDPRPVGNLSIVRTNRAEIPIECRYPRRGNVSS 150

HUMAN      QAILPTWLPFRTTVFSEEKLTFSLRLMEENWNAEKRSPTFHLGDAAHLQA 200
MARMOSET   QAILPTWLPFRTTVFSEEKLTFSLRLMEENWSTEKRTPTFHLGDVAHLQA 200

HUMAN      EIHTGSHVPLRLFVDHCVATPTPDQNASPYHTIVDFHGCLVDGLTDASSA 250
MARMOSET   EIHTGSHVPLRLFVDHCVATPTPDQNASPYHTIVDFHGCLVDGLTDASSA 250

HUMAN      FKVPRPGPDTLQFTVDVFHFANDSRNMIYITCHLKVTLAEQDPDELNKAC 300
MARMOSET   FQAPRPRPDTLQFTVDVFHFANDSRNMIYITCHLKVTLAEQDPDELNKAC 300

HUMAN      SFSKPSNSWFPVEGPADICQCCNKGDCGTPSHSRRQPHVMSQWSRSASRN 350
MARMOSET   SFSKASNSWFPVEGPADICQCCSKGDCGTPSHARRQPHVVSLGSGSPARD 350

HUMAN      RRHVTEEADVTVGPLIFLDRRGDHEVEQWALPSDTSVVLLGVGLAVVVSL 400
MARMOSET   RRHVTEEADVTVGPLIFLDRTGDHEMEQWALPADTSLLLLGTGLAVVALL 400

HUMAN      TLTAVILVLTRRCRTASHPVSASE 424
MARMOSET   TLTAVILVLTRRCRTASLPVSASE 424
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Figure 3. Comparison of the primary amino acid sequences of human (GenBank data base, accession no. M35109) and marmoset ZP3 (Thillai-Koothan *et al*[21]) Potential N-linked glycosylation sites are underlined

Fertility control by immunization with zona pellucida

Antibodies to crude porcine ZP preparations[1] reacted with zona of mouse, rat, rabbit, dog, monkey and human with an increasing degree of cross-reactivity[24]. The immunological cross-reactivity among the various ZP glycoproteins, due to sequence homology has led to the possibility of heterologous immunization. Easy accessibility of porcine ovaries in large numbers from slaughter

Figure 4. Representative photomicrographs showing the ability of MAbs to inhibit binding of boar sperm to porcine oocytes *in vitro*. Monoclonal antibody MA-7 inhibited the binding of sperm to oocyte whereas MA-1 failed to do so

houses made porcine ZP a promising candidate for development of contraceptive vaccine. Polyclonal antibodies against porcine ZP3 have been shown to inhibit porcine sperm–egg interaction[25]. Monoclonal antibodies (MAbs) against porcine ZP3α or ZP3β have been generated in our lab[26–28]. Some of these MAbs inhibit the binding of boar sperm to porcine oocytes *in vitro* (Figure 4). Inhibition in the sperm–egg interaction is not a function of the affinity of the MAbs but dependent on the epitope recognized by these[26]. ZP3α, being the primary sperm receptor ligand in porcine gamete interactions, inhibition of sperm–egg interaction by MAbs to ZP3β may be due to either steric hindrance or ZP3β in its native conformation may also be involved in sperm binding. Interestingly, antibodies against porcine ZP3α and deglycosylated ZP3β (DGZP3β) in addition, also inhibit human gamete interaction[29]. Koyoma *et al.*[30] have shown that MAbs against porcine ZP4 also inhibit the binding of human sperm to human oocytes.

Active immunization with various forms of zona pellucida glycoproteins leads to block in fertility which is either reversible or irreversible. Irreversible infertility, though potentially useful in controlling animal population such as stray dogs, cats, mares[31], poses a major problem in human birth control strategies. Analysis of irreversible fertility reveals that intervention is not at the level of sperm–egg interaction but due to disruption of normal ovarian folliculogenesis. The ovaries showed complete absence of oocytes (primordial and developing) and granulosa/theca cells clusters without oocytes. Lymphocytic infiltration suggested an inflammatory response. Disturbances ranged from mild to severe depending on the species immunized and the zona immunogen used. It was observed for instance that immunization of rabbits with crude ZP preparation led to infertility[32]. This infertility was irreversible and the animals also failed to show a normal follicular maturation in response to superovulation. Ovarian histology showed that the infertility was not due to intervention of sperm–egg interaction but rather to an inhibition of follicular development. Immunization of cynomolgous monkeys with crude porcine ZP antigen rendered them infertile[33]. The irreversibility and abnormal hormonal profiles associated with this immunization was ascribed to contamination of granulosa cell processes in the crude ZP preparations. The immune response so generated was directed against granulosa cell components as well as the zona pellucida leading to extensive ovarian dysfunction eventually culminating in a presentation which was similar to premature ovarian failure. Studies carried out in our laboratory with female bonnet monkeys immunized with a purified porcine ZP3 (55 kDa) glycoprotein as well as ZP3 coupled to the beta-subunit of human chorionic gonadotropin along with adjuvants permitted for human use such as alum, muramyl dipeptide (MDP) and sodium phthalyl derivative of lipopolysaccharide (SPLPS) have indicated that ZP3 immunization with permissible adjuvants could be used for immunocontraception[34]. All animals generated good antibody response and continued to have ovulatory cycles as indicated by the progesterone concentrations, remained infertile in presence of high anti-ZP3 antibody titres and showed no disturbance in cyclicity. Subsequent to decline in the anti-ZP3 antibody titres, out of eight animals immunized, four became pregnant and three continued the pregnancy to term (Table 1). The pups are now 12–18 months old and show normal growth parameters. Ovaries of animals that failed to regain fertility were examined for morphological changes and none of them showed any signs of lymphocytic infiltration or inflammation. Follicles at different stages of development were seen in all the ovaries. No significant reduction in the number of follicles or increase in the number of atretic/degenerating follicles was seen.

Synthetic peptides as possible immunogens

Efficacy of deglycosylated ZP components to block fertility with reduced ovarian dysfunction have promp-

Table 1. Duration of infertility in bonnet monkeys (*Macaca radiata*) immunized with either ZP3 or ZP3-βhCG conjugate

Antigen	Animal number	Duration* of antibody response†	Period of infertility*	Remarks
ZP3	MRA 356	23	42	
100 µg/	MRA 178	9	28	
injection	MRA 283	9	32	
	MRA 293	10	17	Delivered
ZP3-βhCG	MRA 47	17	19	Delivered
100–125 µg/	MRA 49	14	47	
injection	MRA 86	15	47	Aborted
	MRA 130	15	22	Delivered

*Duration in months
†> 500 antibody units.

Table 2. Characteristics of monoclonal antibodies against porcine ZP3α and ZP3β

Characteristics of MAbs	Monoclonal antibody against	
	ZP3α	ZP3β
Carbohydrate/ conformational epitope	28, 7	10
Sequential epitope	420, 421	455, 467, 30
Delay of trypsin mediated zona lysis	420	455, 467, 30
Inhibition of sperm–egg interaction	28, 7, 420	455, 467, 30

ted investigations in the use of ZP sequence-based peptide immunogens as candidates for contraceptive vaccine. This was first demonstrated in a mouse model. Rat MAb against ZP3 capable of blocking fertilization *in vitro* and causing infertility on passive administration[35] was mapped to amino acids 336–342 of the mouse ZP3 sequence. A sixteen amino acid long synthetic peptide (328–342) having this motif and coupled to keyhole limpet haemocyanin was used to immunize randomly bred Swiss mice. Immunization rendered the animals infertile for periods ranging from 1 to 9 months. More importantly, ovarian functions remained normal and following a decline in the antibody titres, the animals regained fertility[36]. Subsequent experiments have shown that the same peptide in complete Freund's adjuvant (CFA) given to (C57B1/6) × (A/J) F1 progeny causes severe autoimmune oophoritis[37]. The oophoritis could be transferred to normal adult mice of the same strain by CD4 + T cell transfer, proving that the ovarian injury was T cell mediated. By synthesizing truncated peptides in the region it was possible to identify B cell epitope (336–342) overlapping with a T cell epitope (330–338).

Work in our laboratory has centered on delineating the epitopes recognized by MAbs generated against porcine ZP3α and ZP3β (Table 2). The nature of the epitopes was determined using enzymatically and chemically cleaved and modified forms of ZP3, and antibodies recognizing conformational and sequential epitopes were identified[27]. The relative distributions of the epitopic domains were determined by performing competitive enzyme-linked immunoassays (ELISAs) and a tentative arbitrary map of these distributions was generated.

Monoclonal antibodies against both ZP3α and ZP3β, and recognizing sequential epitopes, delayed trypsin-mediated zona lysis and also inhibited the binding of ZP3 to sperm membrane vesicle[38]. One of the MAbs against ZP3β (MA-30), capable of inhibiting the porcine sperm–egg interaction *in vitro* was mapped to ~6 kDa fragment of tryptic digest of ZP3. Interestingly, polyclonal antibodies raised against this fragment inhibited the porcine sperm–egg interaction *in vitro*[39]. To precisely localize the minimum binding motif of the MAbs of interest, ZP3β has been synthesized in the form of multiple peptides, 12 aminoacids in length, with an overlap of six aminoacids. These studies are in progress and are beginning to yield interesting results. Using aminoacid substitutions, the contact residues could be further determined.

Concluding comments and future directions

Despite safety concerns regarding disruption of ovarian functions, the ZP remains an attractive target for immunocontraception due to many reasons. The ZP glycoproteins are essential for reproduction and their immunological neutralization will lead to inhibition of fertilization. The developmentally programmed oocyte-specific expression of the ZP proteins rules out cross-reactivity with somatic and germ line cells making their targetting specific. Active immunization will demand generation of anti-ZP antibody titres sufficient to neutralize the zona covering on one egg per cycle. Availability of sequence information and MAbs capable of blocking sperm–egg interaction and recognizing linear epitope will facilitate the delineation of B-cell epitopes which when complexed with the respective antibody will inhibit fertility. Synthetic peptides corresponding to such B-cell domains and devoid of oophoritogenic T-cell epitopes can be employed as candidate antigens.

Synthetic peptides corresponding to promiscuous T-cell epitopes of carrier proteins such as diphtheria toxoid and malaria antigen can provide the necessary T-cell help[40]. Expression of ZP proteins by recombinant DNA route, efficacy of recombinant proteins to regulate fertility[41] will help in designing recombinant vaccines. As new and exciting information on the ZP are forthcoming at a rapid pace, it is hoped that the increase in

our understanding of fertilization process at a molecular level will help in the development of new effective contraceptive strategies.

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