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Comparative Molecular Biology and Immunobiology of Zona Pellucida Glycoproteins: Fundamentals and Applied Aspects for Contraception

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Zona pellucida (ZP), an acellular envelope that surrounds the mammalian oocyte, is composed of three glycoproteins-ZPA, ZPB, and ZPC. The ZP glycoproteins play a crucial role in the initial attachment followed by tight binding of the spermatozoa to the oocyte and subsequent complex cascade of events during fertilization. Due to their critical involvement during fertilization, ZP glycoproteins have been proposed as candidate antigens to develop an immunocontraceptive vaccine for fertility regulation. Towards designing of an immunocontraceptive vaccine for the control of stray dog population and thereby reducing the incidence of rabies, we have successfully cloned and expressed dog ZPA and ZPC in *Escherichia coli*. In addition, bonnet monkey (*Macaca radiata*) ZPA, ZPB, and ZPC have also been cloned, sequenced and expressed in *E. coli* as polyhistidine fusion proteins. Comparison of the deduced amino acid sequence of these three zona proteins showed very high sequence homology with the respective human homologues. Polyclonal antibodies against recombinant bonnet monkey ZPA, ZPB, and ZPC recognized native bonnet monkey and human ZP. Moreover, antibodies against recombinant bonnet monkey ZPB inhibited, *in vitro*, the human sperm-oocyte binding. Simultaneously, attempts have been made to design synthetic peptide immunogens based on the immunologically relevant B-cell epitopes mapped by monoclonal antibodies capable of inhibiting, *in vitro*, sperm-oocyte binding. Availability of recombinant zona proteins and synthetic peptide immunogens will allow us to undertake active immunization studies to determine the prospects of ZP based immunocontraceptive vaccines for fertility regulation.

Key Words: Zona pellucida, Sperm-oocyte binding, Fertility regulation, Immunocontraception, Recombinant zona pellucida glycoproteins, *E. coli* expression, Monoclonal antibodies, B-cell epitope mapping, Synthetic peptide, Immunogenicity

Introduction

The rising global human population (projected to exceed 6.3 billion by the year 2000 AD and to a phenomenal 10 billion by the year 2050 AD) is an issue of concern. Another collateral issue is the regulation of wildlife population. On one hand, it is an alarming situation where many species are on the verge of extinction and on the other hand, there is an unchecked increase in the population of some of the species such as stray dogs. The latter situation is further accentuated by the new legislations and awareness in the society that forbid killing of these animals. Hence, there is a need to develop new strategies by which one can control wild life as well as human population.

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Abbreviations: *aa*, amino acid(s); *bp*, base pair(s); *bmZPA*, bonnet monkey zona pellucida glycoprotein-A; *bmZPB*, bonnet monkey zona pellucida glycoprotein-B; *bmZPC*, bonnet monkey zona pellucida glycoprotein-C; *cDNA*, complementary deoxyribonucleic acid; *DT*, diphtheria toxoid; *dZPA*, dog zona pellucida glycoprotein-A; *dZPC*, dog zona pellucida glycoprotein-C; *kb*, kilobase(s) or 1000 base pair(s); *kDa*, kilodalton; *MAb*, monoclonal antibody; *Ni-NTA*, nickel-nitrilotriacetic acid; *nt*, nucleotide(s); *PAGE*, polyacrylamide gel electrophoresis; *PCR*, polymerase chain reaction; *r*, recombinant; *SDS*, sodium dodecylsulfate; *ZP*, zona pellucida

Immunocontraceptive vaccines have been proposed as a means to achieve fertility regulation. It will entail generation of humoral and/or cell mediated immune response against antigens that have crucial role in the process of reproduction. An ideal immunocontraceptive vaccine for humans should be (i) effective in preventing conception, (ii) potentially reversible, (iii) free of any side effects, and (iv) reliable, affordable and easy to administer. For wild life control, a vaccine leading to irreversible block in fertility may also be acceptable and in some situations desirable. Vaccines have been traditionally used as an approach of "herd immunization". It is desirable that contraceptive vaccines meant for humans, ideally generate an effective immune response in 100% of the recipients. However, it will not be an absolute requirement for vaccines meant for controlling wildlife population. An immunocontraceptive vaccine meant for female animals and having an efficacy of say 50-80% will also be useful in controlling the wildlife population. Such vaccines can be designed to interfere at various stages of reproduction such as (i) gametogenesis, (ii) sperm-oocyte binding, and (iii) post-fertilization stage. Moreover, there is also a need to devise and optimize strategies for *in vitro* fertilization and embryo transfer to propagate the species that are in danger of extinction.

To design an immunocontraceptive vaccine, aiming to interfere at the level of sperm-oocyte interaction, it is imperative to understand the structure and functions of the molecules involved in gamete interaction. The mammalian oocyte is surrounded by an acellular translucent envelope termed the zona pellucida (ZP), which is comprised of three distinct glycoproteins. ZP glycoproteins serve as the docking site for species-specific sperm binding, induce bound spermatozoa to undergo the acrosome reaction, and affect block to polyspermy (Snell & White 1996). Various putative zona receptor molecules on spermatozoa have been characterized (Gupta et al. 1997). In this article, characteristics of ZP glycoproteins, their involvement during fertilization and various approaches being developed to use these as immunogens for development of fertility regulating vaccines will be reviewed.

Zona Pellucida Glycoproteins

ZP, a translucent acellular envelope that surrounds the mammalian oocyte, is comprised of three biochemically and immunologically distinct glycoproteins, which have been classified, based on their mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as ZP1, ZP2 and ZP3. ZP glycoproteins isolated from different species, inspite of having very similar size of the polypeptide core, migrate differently on SDS-PAGE due to the differences in post-translational modifications. For example, mouse and hamster ZP3 sharing a sequence homology of ~82% have very different apparent molecular weight of ~83 kDa and ~56 kDa respectively (Moller et al. 1990). Our group has used highly specific antibody probes generated against recombinant (r) bonnet monkey (*Macaca radiata)* ZP1, ZP2 and ZP3 to characterize human (h) ZP glycoproteins in Western blot. Under non-reducing condition hZP1, hZP2 and hZP3 resolved as 60, 100 and 53 kDa bands respectively (Gupta et al. 1998). These studies conclusively showed that in the previous studies hZP2 was likely to have been misinterpreted as being ZP1. To circumvent this problem, recently ZP glycoproteins have also been classified on the basis of the size of their mRNA transcript as ZPA, ZPB and ZPC where ZPA is the longest and ZPC is the smallest (Harris et al. 1994). In the present manuscript, we have followed the latter classification.

Genomic Organization of ZP Glycoproteins

The genes for ZP glycoproteins have been cloned and sequenced from several species. Human ZPA (hZPA) gene spans 13 kb having 19 exons and is transcribed into a 2235 nucleotides (nt) mRNA encoding a polypeptide of 745 amino acid (aa) residues (Liang & Dean 1993). Human ZPB (hZPB) gene spans 11 kb and is comprised of 12 exons. The hZPB transcript has an open reading frame (ORF) of 1620 nt and encodes a

Table 1 *Salient Features of the Bonnet Monkey ZPA, ZPB and ZPC*

Zona Proteins	Open Reading Frame (ORF)	Length of polypeptide	N-terminal signal sequence	C-terminal domain after furin cleavage site	% Identity at aa level with mouse	human
ZPA	2235nt	745aa	1-38 aa	642-745 aa	70.3	94.2
ZPB	1617nt	539aa	1-21 aa	467-539 aa	36.9	92.0
ZPC	1272nt	424aa	1-22 aa	353-424 aa	67.2	93.9

polypeptide of 540 aa residues (Harris et al. 1994). Human ZPC (hZPC) gene spans 18.3 kb containing 8 exons encoding a polypeptide of 424 aa residues (Chamberlin & Dean 1990).

Towards the development of a ZP based immunocontraceptive vaccine for humans, and to mimic the situation in a closely related species, our group has been working on establishing a non-human primate model. For this purpose we have used bonnet monkey (*M. radiata*), a primate close in evolution and reproductive physiology to humans. Furthermore, it is native to South India and its reproductive physiology and endocrinology has been reasonably well established. We have successfully cloned and sequenced the complementary deoxyribonucleic acid (cDNA) for bonnet monkey (bm) ZPA (bmZPA), ZPB (bmZPB) and ZPC (bmZPC) (Kolluri et al. 1995, Gupta et al. 1997, Jethanandani et al. 1998). Comparison of the deduced aa sequence of bmZPA, bmZPB and bmZPC revealed high sequence identity with respective human homologue as compared to the mouse homologue (table 1). bmZPA has 22 cystein residues as compared to 20 in hZPA and the position of majority of these within the sequence is conserved suggesting thereby an overall similar tertiary structure. The major difference between bmZPB and hZPB is the deletion of 100-127 aa domain in bmZPB (Gupta et al. 1997). In mouse model, the domain corresponding to 318-352 aa of ZPC has been implicated in sperm-zona interaction. Interestingly, high aa sequence identity of this domain between hZPC and bmZPC is maintained.

The information available so far on the zona genes and deduced aa sequence of the respective zona proteins suggests that the three zona proteins share certain common structural motifs. Each has a N-terminal signal peptide, which directs these into a secretory pathway and is later cleaved off from the mature polypeptide chain. Each of the zona proteins also has a transmembrane-like domain near its C-terminus about 34-47 aa downstream of a potential tetra basic furin cleavage site, R-X-R-R. Proteolytic processing of this hydrophobic transmembrane-like domain may play a role in intracellular trafficking of these secreted proteins or in their interaction in the extracellular matrix.

Sequence Homology Among ZP Glycoproteins

Comparison of the deduced aa sequence of a given zona protein from several species reveal a variable degree of sequence identity. Is there any similarity in the aa sequence among ZPA, ZPB and ZPC? Comparison of the deduced aa sequence of bmZPA vs bmZPC and bmZPB vs bmZPC revealed no significant identity. However, considerable identity at the C-terminal end was observed between bmZPA and bmZPB. The region from aa residues 369 to 418 of bmZPB has 52% identical aa residues as compared to bmZPA and also revealed two stretches of 5 continuous identical aa residues. Figure 1 represents some of the selected stretches of bmZPA showing high aa sequence identity with bmZPB. It might insinuate that these two proteins might have originated from a common ancestral gene and may have diversified further through gene duplication or recombination.

Zona Pellucida Proteins	Selected Regions Showing High Sequence Identity
bmZPA	455 R D S **E** F R **M** **T** V **K** C S Y S 468
bmZPB	275 R D S **I** F R **L** **H** V **S** C S Y S 288
bmZPA	514 Y P **L** V **R** **F** L R **Q** P I Y **M** E V 528
bmZPB	336 Y P **V** V **K** **L** L R **D** P I Y **V** E V 350
bmZPA	552 D P **D** S **F** P Q W **N** **I** V V **D** G C **A** Y **E** **L** D N Y Q T 575
bmZPB	374 D P **L** S **Q** P Q W **P** **I** **L** V **K** G C **P** Y **I** **G** D N Y Q T 397
bmZPA	587 P **D** H Y Q R F **D** **M** **K** **A** F **A** F V 601
bmZPB	411 P **S** H Y Q R F **S** **I** **F** **T** F **S** F V 425

Figure 1 Homology of the deduced aa sequence of bmZPA with bmZPB. Deduced aa sequence of bonnet monkey ZPA and ZPB were analyzed for aa sequence identity by palign using PCGENE. Selected stretches of the ZPA showing highest aa identity with ZPB are shown. Amino acid residues are numbered from the translational start codons. Dissimilar aa are shown in bold.

Conservation of ZP Glycoproteins through Evolution

Evidence that the ZP glycoproteins are conserved not only through mammalian evolution but also through the entire vertebrate evolution has been accumulating. Cloning and sequencing of the vitelline envelope (VE) glycoproteins, which surrounds the plasma membrane in fish oocytes has revealed considerable homology with the mammalian ZP proteins. The primary structure of medaka (*Oryzias latipes*) egg VE polypeptide called L-SF41 was found to be significantly similar to the mammalian ZPC polypeptide (Murata et al. 1995). Moreover, 10 of the invariant Cys residues have been conserved in this species indicating the overall conservation of the three-dimensional structure of the ZP family through evolution. In the flounder, *Pseudopleuronectes americanus,* the gene encoding another VE protein called wf_{female} shares considerable sequence homology with the ZPA protein family. Analysis of the sequence has revealed that exons 11-16 in the mouse and exons 2-7 in the fish wf_{female} gene have been derived from a common ancestor (Liang et al. 1990, Lyons et al. 1993). The fishes are the oldest of the vertebrates and the observed similarities between the ZP and VE polypeptides bears directly on the evolution of the ZP proteins. However, in teleost fish (*Oryzias latipes*), unlike the ZP glycoproteins, precursors of the VE are synthesized in the liver and transported to the growing oocytes. Comparison of the deduced aa sequence of the marsupial *Trichosurus vulpecula* (Brushtail possum) ZPB with seven species of eutherian mammals (human, macaque, marmoset, pig, cat, rabbit and mouse) and *Xenopus* revealed high level of sequence similarity between species and out of 20 cysteine residues, 18 are found to be conserved across all species (Haines et al. 1999).

In the anuran, *Xenopus laevis,* homologues of the ZP glycoproteins ZPA, ZPB and ZPC share extensive homology with human, porcine and mouse sequences (Hedrick 1996). Comparison of the translated aa sequences of the *Xenopus* ZPA gene with the human, porcine and mouse revealed identities of 28.5%, 27.6% and 26.9% respectively. The ZPB and ZPC genes are relatively better conserved with identities of 41.6%, 41.7% and 36.8% with human, porcine and mouse homologous ZPB sequences and 40.9%, 40.0% and 40.8% with the human, porcine and mouse homologous ZPC sequences respectively.

Is Expression of ZP Glycoproteins Oocyte Specific?

In mouse model, using a variety of experimental procedures, it has been demonstrated that the ZP glycoproteins are exclusively expressed in

the oocyte (Ringuette et al. 1986, Liang et al. 1990, Epifano et al. 1995). However, *in situ* hybridization of cynomolgous monkey ovaries with digoxigenin-labeled specific cDNA probe revealed the presence of mRNA encoding for ZPA in growing follicles at all stages and also in the granulosa cells of mature preovulatory follicles (Martinez et al. 1996). Transcript for ZPB is present in the secondary and tertiary follicles and absent in primordial, primary and antral follicles as well as granulosa cells. Recent, *in situ* hybridization studies in *T. vulpecula* (Brushtail possum) also revealed the absence of ZPB from granulosa cells (Haines et al. 1999). ZPC is present in oocytes at all stages of folliculogenesis as well as granulosa cells (Martinez et al. 1996). Presence of ZPC on primordial follicles and granulosa cells of the human ovaries has also been demonstrated by using specific polyclonal as well as monoclonal antibodies (Grootenhuis et al. 1996). These observations are at variance with the ones from mouse model and require further clarification on the possibility of expression of ZP glycoproteins by other ovarian associated cells such as granulosa cells in addition to the oocyte.

Regulation of Expression

In the mouse model, as the oocytes grow, three zona transcripts accumulate and represent ~ 1.5% of the total poly(A)$^+$ RNA in the 50-60 μm oocyte. Of the three, ZPB is least abundant of the transcripts and represents only 25% of the ZPA and ZPC, which are present in roughly equimolar amounts. Post ovulation, the oocytes have less than 5% of the peak amounts of the zona transcripts. The concurrent accumulation of the ZP transcripts suggests that common transcriptional regulatory events may be involved in the regulation of gene expression.

Further analysis of the upstream sequences of mouse and human ZPA and ZPC gene reveals the presence of short conserved DNA sequences upstream of the TATA box (I, IIA, IIB, III, and IV). Mutational analysis has revealed that the 12 bp element IV is both necessary and sufficient for high level expression of a reporter gene in mouse oocytes. Oligonucleotides corresponding to the conserved upstream regulatory elements from either ZPA/ZPC form DNA-protein complexes of identical mobility in gel retardation assays indicating the involvement of similar regulatory processes in expression (Millar et al. 1991). A putative transcription factor, zona pellucida gene activating protein-1 (ZAP-1) binding to the conserved element IV has been implicated in the positive regulation of ZP expression. The onset of mouse ZPA transcription as well as the profile of its subsequent accumulation correlates with the ZAP-1 DNA binding activity (Millar et al. 1993). An oocyte specific 60 kDa protein (OSP-1), binding to nucleotides –99 to –86 of the mouse promoter, has also been identified and has been proposed as an oocyte specific transcription factor (Schickler et al. 1992). By effecting an inhibition of the *de novo* biosynthesis of zona proteins by antisense oligonucleotides, it was established that abolishment of either ZPA or ZPC protein synthesis, prevented the incorporation of the other protein into the extracellular zona matrix suggesting that ZPA and ZPC proteins are independent of each other in their biosynthesis but are dependent upon each other for incorporation into the zona matrix (Tong et al. 1995).

Functions of ZP Glycoproteins

The ZP glycoproteins mediate the initial recognition and binding of spermatozoa to oocyte in a species-specific manner and induce bound sperm to undergo acrosome reaction. The acrosome reacted spermatozoa penetrates the ZP and enters the perivitelline space where spermatozoa membrane fuses with the oolemma, the plasma membrane of the oocyte (Snell & White 1996). Fusion triggers a rapid electrical depolarization of the egg membrane followed by the cortical reaction. Cortical reaction induces changes in sperm-adhesive glycoproteins within ZP matrix resulting in an inability of the oocyte to bind fresh sperm and also make ZP matrix resistant to subsequent penetration by acrosome reacted spermatozoa, thereby blocking polyspermy. Moreover, ZP also protects cleaving embryos as they traverse through the female reproductive tract prior to implantation.

(a) Binding of Sperm

(i) Role of ZPC and other zona glycoproteins

In mouse, ZPC purified from ovulated eggs can bind capacitated sperm and induce acrosome reaction in bound spermatozoa (Bleil & Wassarman 1986). Employing recombinant ZPC glycoprotein, it has further been confirmed that in the mouse, hamster and human, ZPC serves as the primary sperm receptor (Bleil & Wassarman 1988, Moller et al. 1990, van-Duin et al. 1994). However, ZPC does not bind to acrosome reacted sperm. The fact that ZPC plays a crucial role during fertilization is further confirmed by the observations that the mice with homozygous mutant ZPC gene failed to conceive on mating with male mice of proven fertility (Rankin et al. 1996, Liu et al. 1996). However, recent studies in pig model showed that high molecular weight hetero-complexes of ZPC with ZPB bind with very high affinity to boar sperm thereby suggesting that both the glycoproteins participate in the sperm binding process (Yurewicz et al. 1998). Similarly, in rabbits, both the 55 (ZPB) and 45 (ZPC) kDa zona glycoproteins have been shown to bind to sperm receptor (Yamasaki et al. 1995). Further studies employing either recombinant zona proteins/glycoproteins or those purified from native source will clarify whether ZPC alone or the interaction of more than one zona glycoproteins is a prerequisite for ZP matrix to act as a primary receptor.

(ii) Role of carbohydrates

Experiments using enzymatically digested purified ZPC and incubation of resulting fragment with capacitated sperm has revealed that in mice, O-linked oligosaccharides present on ZPC plays an important role in recognition and binding of sperm to oocyte. Subsequently, it was demonstrated that terminal galactose residues in α or β linkages as well as N-acetylglucosamine in β linkage of O-linked oligosaccharides on ZP3 are critical (Wassarman & Litscher 1995). However, mice deficient in glycosyl transferase, which amends terminal galactose in α-linkage, are fully fertile (Thall et al. 1995) suggesting that either galactose in β-linkage or N-acetylglucosamine (or both) is critical residue. In porcine model, both O-linked oligosaccharides (Yurewicz et al. 1991) and N-linked oligosaccharides (Nakano et al. 1996) have been implicated in sperm-egg binding.

(b) Induction of the Acrosome Reaction

In addition to the initial recognition and binding of spermatozoa during fertilization, the ZP in many species, excepting the guinea pig, is the stimulus for the acrosome reaction. In mice, ZPC has been identified as the acrosome reaction-inducer. Acrosome reaction leads to (i) release of proteolytic enzymes such as hyaluronidase, phosphatases, glycosidases, lipases and proteases required for sperm to penetrate the ZP matrix and (ii) remodeling of sperm surface for continued adhesion to ZP as well as fusion with the egg membrane (Yanagimachi, 1994). The acrosome reaction is characterized by influx of Ca^{++} and Na^{+} and efflux of H^{+} leading to an increase in the intracellular *p*H. ZPC induces the activation of a G-coupled adhesion molecule, which may lead to production of inositol triphosphate (IP3), and subsequent activation of a voltage gated Ca^{++} channel in the sperm plasma membrane (Snell & White 1996). Progesterone may also act in a synergistic fashion with ZPC in the induction of the acrosome reaction (Roldan et al. 1994).

Recently, it has been demonstrated that human spermatozoa when incubated, *in vitro*, in presence of recombinant hZPC expressed in *E. coli* undergo acrosome reaction, thereby suggesting that the presence of carbohydrates on ZPC polypeptide backbone may not be an absolute requirement for ZPC to induce acrosome reaction (Chapman et al. 1998). Further studies employing recombinant zona proteins expressed either in prokaryotic or eukaryotic expression systems will help in delineation of the importance of carbohydrates in sperm-oocyte interaction and induction of acrosome reaction.

(c) Binding of Acrosome Reacted Spermatozoa and Avoidance of Polyspermy

Subsequent to the acrosome reaction, ZPA acts as a secondary receptor and helps in the maintenance of acrosome reacted spermatozoa

binding to the oocyte. The ligand for ZPA on the sperm inner acrosomal membrane can be either a homologue of PH20 (a guinea pig sperm surface protein) or acrosin (pig) (Primakoff et al. 1985, Yonezawa et al. 1995). In mouse model following fertilization, proteolysis of ZPA as a result of cortical reaction, results in the formation of small molecular weight fragments, which do not dissociate but remain non-covalently bound (Wassarman 1987). This increase in non-covalent interactions makes ZP resistant to proteolytic cleavage and acts as a protective barrier for polyspermy. Concomitantly, it is also associated with the loss of ZPC acting as a sperm receptor. The precise role of ZPB is not clear, but probably it is acting as a cross-linker between ZPA and ZPC.

Potentials of ZP Glycoproteins as Candidate Antigens for Immunocontraception

By virtue of their critical involvement in fertilization, these have been proposed as candidate antigens to develop an immunocontraceptive vaccine. Antibodies generated against the ZP glycoproteins purified from a given species showed variable degree of immunological cross-reactivity with ZP from other species. The immunological cross reactivity among ZP glycoproteins from different species is due to variable degree of aa sequence homology and this property has allowed the possibility of heterologous immunization. Initial studies were carried out with pig ZP glycoproteins due to (i) availability of a large number of pig ovaries from abattoirs, (ii) immunological cross reactivity of antibodies thus generated with ZP from various species including non-human primates and humans, and (iii) demonstrated *in vitro* effect of polyclonal antibodies against porcine ZP glycoproteins to inhibit porcine and human sperm-egg binding.

Control of Animal Population

Porcine ZP glycoproteins have been tested for immunocontraception in several wild species. In feral horse and donkey populations a single annual booster was enough to prevent conception, without affecting the complex social behaviour of the animals. Short term treatment for up to 4 consecutive years did not result in any detectable debilitating side effects and contraceptive effect was reversible while long term treatment (5-7 years) was associated with few ovulation failures and depressed urinary estrogen levels (Kirkpatrick et al. 1990). Seventy four species of captive zoo animals have been tested with the porcine ZP glycoproteins based vaccine with documented success in 27 out of the 28 species for which the data are available (Kirkpatrick et al. 1996).

In addition to conventional immunization, various other strategies have also been employed to deliver the ZP glycoproteins such as viral vectors or liposome. Ectromelia virus, a natural pathogen for mice that causes mousepox was used as live vector. A recombinant ectromelia virus expressing entire mouse ZPC c-DNA was generated (Jackson et al. 1998). Immunization of a group of mice with recombinant viruses lead to decrease in fertility as well as litter size compared to the group immunized with virus containing only the plasmid. Immunization lead to disruption of follicular development but did not cause any ovarian oophoritis. The group immunized with the recombinant virus showed milder symptoms and less mortality as compared to the mice immunized with wild type viruses. A single shot of porcine solubilized zona pellucida using liposome delivery system decreased the pup production in the grey seal (*Halihoerus grypus*) by 90% over a period of 5 years (Brown et al. 1997).

Control of Stray Dogs Population

Active immunization of bitches with crude porcine zonae pellucidae induced infertility (Mahi-Brown et al. 1982). Immunization resulted in abnormal estrus cycles characterized by prolonged proestrus or estrus. Oocytes recovered from the ovaries of the immunized animals failed to bind sperm. Histological examination of the ovaries revealed that animals with the highest antibody titres showed depletion of the oocytes. In order to generate an adequate antibody response, a variety of adjuvants have been used. Earlier studies have

Table 2 *Comparison of Deduced Amino Acid Sequence of Dog ZPA and ZPC with other Species*

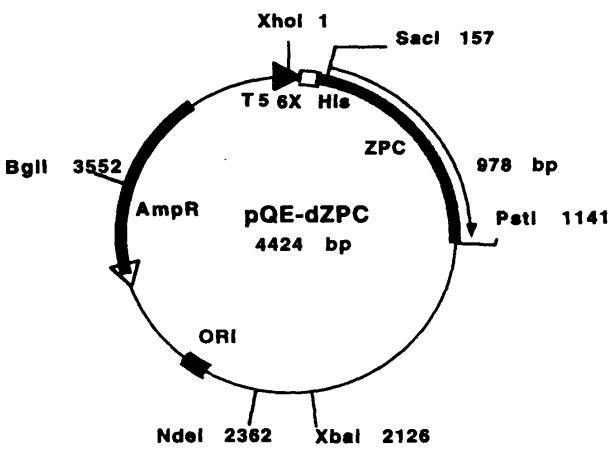
	Percent identity of deduced amino acid sequence with respective zona pellucida protein from different species						
	Dog	Mouse	Rabbit	Pig	Cat	Bonnet monkey	Human
ZPA	100	57.9	66.2	69.6	83.1	50.0	67.5
ZPC	100	64.7	65.3	75.7	79.2	67.4	70.1

employed Freund's complete adjuvant (CFA). However, the induction of granulomatous lesion at the site of injection renders it unsuitable for use in humans including several animal species. Immunization of female dogs with porcine zonae pellucidae adsorbed on alum along with CP20, 961, a synthetic lipid amine, as an emulsifier resulted in variable antibody response (Mahi-Brown et al. 1985). One of the animals developed a temporary inflammatory response to the adjuvants at the site of injection, developed the lowest antibody response, and conceived when bred, while 3 others produced higher antibody titres, remained infertile and did not show any inflammatory response. Hence by employing proper adjuvants and purified ZP glycoproteins (devoid of other ovary associated proteins) it is possible to block fertility of stray dog with minimal acceptable side effects.

In India, increasing population of stray (wild) dogs is a significant problem. It is also associated with high incidence of rabies. We are exploring the possibility of designing an immunocontraceptive vaccine based on ZP glycoproteins, in an effort to control the population of stray dogs. This effort will demand the availability of large amounts of purified dog zona proteins without contaminants of any other ovarian associated proteins. Immunization with dog zona proteins *per se* may generate optimum immune response with better recognition of native dog ZP.

In order to express dog ZPC (dZPC) by DNA recombinant technology, an internal fragment (978 bp) excluding the N-terminal signal sequence and the C-terminal transmembrane-like domain was amplified by polymerase chain reaction (PCR) and cloned in-frame downstream of the T5 promoter under *lac* operator control in the pQE-30 vector (Santhanam et al. 1998). dZPC was expressed in *E. coli* as polyhistidine fusion protein for its convenient purification using Ni-NTA resin. In Western blot, the recombinant dZPC (r-dZPC) revealed a major band of 42 kDa (figure 2). In order to obtain higher yields of r-dZPC, a process of fed batch fermentation (3.5 L capacity) has been optimized. Batch fermentation yielded 30 mg/L of r-dZPC as compared to 4 mg/L from a shake flask culture.

Recently, our group has also succeeded in the expression of dZPA as polyhistidine fusion protein in *E. coli* (unpublished observations). Comparison of deduced aa sequence of dZPA and dZPC with their respective homologues from other species is summarized in table 2 (Harris et al. 1994). Both dZPA and dZPC showed highest aa sequence identity with the respective homologues from cat. High antibody titres were observed in male rabbits immunized with r-dZPC (Santhanam et al. 1998). The immunogenicity of the r-dZPC was also evaluated in the female dogs. For this purpose r-dZPC was conjugated with DT using glutraldehyde and female dogs immunized with r-dZPC-DT conjugate led to the generation of high antibody titres against r-dZPC (Santhanam et al. 1998). Furthermore the antibodies generated against r-dZPC recognized the native dog ZP. Active immunization studies in stray female dogs with r-dZPC-DT and r-dZPA-DT conjugates are underway to test their efficacy in blocking fertility.



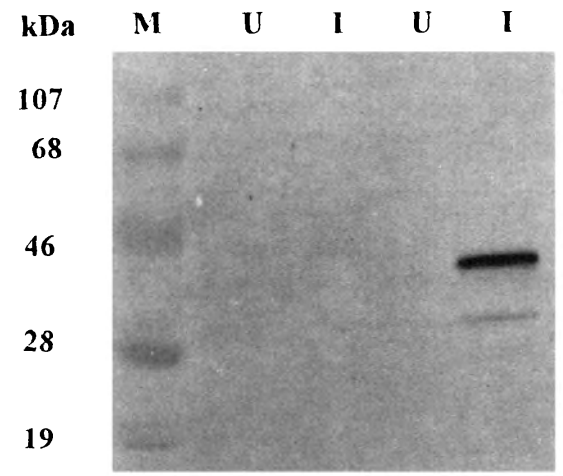


Figure 2 Expression of dZPC in **E. coli**. Schematic representation of the construction of r-dZPC in pQE-30 expression vector is shown in the left panel. PCR amplified dZPC cDNA fragment (79-1056 nt) excluding the signal sequence and transmembrane-like domain was cloned downstream of T5 promoter in the pQE-30 vector. Right panel represents the immunoblot of bacterial cell lysates harboring the pQE-dZPC plasmid. The first two lanes represent wild type of host cells without pQE-dZPC plasmid. T5, promoter of phage T5; AmpR, ampicillin resistance marker; ORI, origin of replication; Lane M, molecular weight markers; U-uninduced cells, I-induced cells with isopropyl-β-D-thiogalactopyranoside.

Immunocontraceptive Vaccine for Humans

Immunization of female rabbits or non-human primates with purified native or deglycosylated porcine ZP glycoproteins leads to a block of fertility (Skinner et al. 1984, Dunbar et al. 1989, Sacco et al. 1989, Jones et al. 1992, Paterson et al. 1992, Bagavant et al. 1994). However, it is invariably associated with either a transient or irreversible alteration in the cyclicity, hormonal profile, and follicular development in the ovary (Skinner et al. 1984, Dunbar et al. 1989, Paterson et al. 1992). These changes have been attributed to the following factors:

(i) Presence of other ovarian associated proteins in addition to the ZP glycoproteins

(ii) Presence of oophoritogenic T cell epitopes in the ZP glycoproteins

(iii) Nature of adjuvants employed

To address some of these issues our group has followed-up the following strategies:

(a) Development of a Non-human Primate Model

The major bottle-neck in the development and critical evaluation of an immunocontraceptive vaccine for human application is non-availability of a suitable animal model. In order to fill-up this gap, with special reference to ZP glycoproteins based immunocontraceptive vaccine, we have established bonnet monkey-a non-human primate as an animal model. Highest observed deduced aa sequence homology of bmZPA, bmZPB, and bmZPC with their homologues from human further reiterate our contention about its suitability as an animal model to rigorously test not only the efficacy of immunization with bonnet monkey ZP proteins/ glycoproteins to block fertility but also detailed and careful analysis of any autoimmune side effects following such an approach.

(b) Cloning and Expression of bmZPA, bmZPB, and bmZPC

Bonnet monkey ZP glycoproteins-bmZPA, bmZPB, and bmZPC have been expressed in *E. coli* as polyhistidine fusion proteins so as to allow their convenient purification. The cDNA corresponding to bmZPA, bmZPB, and bmZPC excluding the N-terminal signal sequence and the C-terminal domain after furin cleavage site that included transmembrane-like domain were PCR amplified. The amplified fragments after

Table 3 *Characteristics of the Recombinant Bonnet Monkey ZP Proteins Expressed in E. coli*

Recombinant protein	Size of major bands recognized in Western blot (kDa)	Reactivity of polyclonal antibodies in an indirect immunofluorescence assay with		Effect of polyclonal antibodies to inhibit human sperm oocyte binding
		Human ZP	Bonnet ZP	
ZPA	68	Positive	Positive	Not tested
ZPB	51, 40	Positive	Positive	Positive
ZPC	50	Positive	Positive	Not tested

digestion with appropriate restriction enzymes were cloned in frame downstream of the T5 promoter under *lac* operator control for expression in the pQE-30 vector as described previously (Kaul et al. 1997, Gupta et al. 1997, Jethanandani et al. 1998). SDS-PAGE and Western blot analysis revealed the major bands of 68 kDa for bmZPA, 51 and 40 kDa for bmZPB, and 50 kDa for bmZPC (table 3). Expression of bmZPC is further optimized to reduce the level of premature translation termination products and to obtain higher yield of recombinant protein. Use of protease deficient host cells such as *E. coli* strain BL21(pLysS) helped in reducing the level of premature translational termination products (Srivastava et al. 2000). Further, a high cell density batch fermentation process was developed whereby 50 mg of the purified r-bmZPC was obtained from a liter of fermentation broth as compared to 4 mg/L of shake flask culture (Srivastava et al. 2000).

(c) Immunological Characterization of Recombinant Bonnet Monkey ZP Proteins

Polyclonal antibodies generated against r-bmZPA, r-bmZPB, and r-bmZPC in male rabbits recognized the native bonnet monkey and human ZP (Kaul et al. 1997, Gupta et al. 1997, Jethanandani et al. 1998, Gupta et al. 1998) (table 3). Furthermore antibodies against r-bmZPB inhibited, *in vitro,* the binding of human spermatozoa to antibody treated zona encased human oocytes (Govind et al. 2000) (table 3). To test the contraceptive efficacy of recombinant bonnet monkey ZP proteins, r-bmZPA, r-bmZPB, and r-bmZPC have been conjugated to DT. Immunization of female bonnet monkeys with r-bmZPA-DT, r-bmZPB-DT, and r-bmZPC-DT conjugates using adjuvants permissible for human use lead to generation of the antibodies against the respective recombinant zona protein and DT (unpublished observations).

(d) Delineation of Immunologically Relevant B-cell Epitope

An alternate to recombinant proteins is to use defined synthetic peptides as immunogens. Reversible block of fertility was reported in Swiss mice immunized with 15 mer peptide corresponding to mouse ZPC and coupled to keyhole limpet haemocynin (Millar et al. 1989). Subsequently, this peptide in complete Freund's adjuvant given to (C57Bl/6)x(A/J) F1 progeny caused severe autoimmune oophoritis (Rhim et al. 1992). A B-cell epitope (336-342 aa) overlapping with a T-cell epitope (330-338 aa) was identified by synthesis of truncated peptides. Studies with 13-mer peptide (330-342) led to the identification of a minimal T-cell epitope (330-337 aa, NSSSSQFQ) required for disease induction. Alanine substitution along this sequence helped in the identification of residues important either for T-cell binding or MHC recognition (Lou et al. 1993, Garza & Tung 1995). The peptide induced T-cell mediated oophoritis resolved spontaneously after 4 months and the recovered ovary showed both primordial and developing follicles with no lymphocyte infiltration (Lou et al. 1995a). They were also resistant to the reinduction of the

Table 4 *B-cell Epitopes Mapped by Monoclonal Antibodies Capable of Inhibiting Sperm-Oocyte Binding*

Monoclonal Antibody	Specificity	*In vitro* inhibition of binding of	Mapped epitope
MA-412	Porcine ZPB	Porcine sperm-oocyte	TSPPLLWDSVHL (aa 205-216)
MA-420	Porcine ZPB	Porcine sperm-oocyte	FLALDVPTIGLC (aa 113-144)
MA-30/ 455/ 467	Porcine ZPC	Porcine sperm-oocyte	QPVWQDEGQRLL (aa 23-34)
MA-809/ 811/ 813/ 825	bmZPB	Human sperm-oocyte	DAPDTDWCDSIP (aa 136-147)

disease. Further studies were done using chimeric synthetic peptides encompassing the minimal B-cell epitope (335-342 aa, QFQIHGPR) with the T-cell cross reactive phenylalanine substituted by alanine. The T-cell help was provided by a "promiscuous" T-cell epitope of bovine RNase co-linearly synthesized with the B-cell epitope of mouse ZPC. This peptide elicited anti-ZP antibodies in mice of 8 different H-2 haplotypes without activation of oophoritogenic T-cells (Lou et al. 1995b). This showed a way by which the MHC driven non responsiveness to a self antigen can be overcome and the self pathogenic T-cell responses can be avoided.

Earlier studies in our laboratory focussed on mapping the B-cell epitopes recognized by monoclonal antibodies (MAbs) generated against porcine ZPB and ZPC and capable of inhibiting, *in vitro,* the porcine sperm-oocyte binding (Bagavant et al. 1993, Gupta et al. 1995). Using multipin peptide synthesis approach, MAbs, MA-412 and MA-420 having specificity for porcine ZPB were mapped to aa 205-216 and aa 133-144 respectively (Gupta et al. 1996) (table 4). Using similar strategy the MAbs, MA-455, MA-467 and MA-30 having specificity for porcine ZPC were mapped to aa 23-34 (Gupta et al. 1995, Afzalpurkar & Gupta 1997) (table 4). A cocktail of immune sera generated against the porcine ZPC synthetic peptides designed based on B-cell epitope mapping data, inhibited *in vitro* porcine sperm-oocyte binding (Afzalpurkar et al. 1997a). Polyclonal antibodies against synthetic peptide corresponding to bmZPC aa 324-347 also significantly inhibited human sperm-oocyte binding (Afzalpurkar et al. 1997b). Active immunization studies with bmZPC peptide conjugated with DT in female bonnet monkeys are in progress. The epitope for MAb (MAb-5H4) generated against porcine ZP4 (a fragment of ZPA) and capable of inhibiting porcine and human sperm-egg binding, was mapped to aa 50-67 (Koyama et al. 1999). Auto- and hetero -antibodies produced in rabbits and mice against a synthetic rabbit 18 mer peptide corresponding to the above sequence showed a strong blocking effect on fertilization in humans.

Four MAbs against r-bmZPB and capable of inhibiting, *in vitro,* human sperm-oocyte binding, recognize an epitope corresponding to aa 136-147 (Govind et al. 1999) (table 4). The epitopic domain corresponding to aa 136-147 of bmZPB is completely conserved in hZPB. Based on the data generated in our laboratory and that of the others, we have recently designed a cDNA construct by PCR, encompassing the B-cell epitopes of bmZPA, bmZPB, and bmZPC separated from each other by a tri-glycine spacer. The cDNA have been cloned in prokaryotic expression vector and the recombinant protein expressed in *E. coli* (unpublished results). The studies to test the efficacy of the recombinant protein encompassing the B-cell epitopes of the bonnet monkey three zona proteins to generate antibodies that may recognize bonnet monkey and human ZP and inhibit, *in vitro,* human sperm-oocyte binding are in progress.

Future Investigations

Attempts will be made to purify r-bmZPA, r-bmZPB, and r-bmZPC in renatured form to understand more precisely their functions during fertilization especially in primates. Expression of these in glycosylated form by using yeast, baculovirus, or mammalian expression systems

will further help in delineation of the role of carbohydrate moieties in the interaction of the zona proteins to sperm receptor. Better understanding of the fertilization *per se* will help in optimizing the conditions to achieve *in vitro* fertilization followed by embryo transfer to propogate the endangered species. Active immunization studies with recombinant proteins conjugated to suitable carrier in female bonnet monkeys will help in elucidating the potential of zona pellucida based candidate antigens for design of an immunocontraceptive vaccine for human application. The bonnet monkey as an animal model will help in careful analysis of autoimmune side effects, if any, following such an immunization regimen. To control the population of stray dogs, instead of conjugating r-dZPC/ZPB with DT, we are planning to express hybrid protein in *E. coli* consisting of dZPC/dZPB linked with rabies glycoprotein G. Such an immunogen will provide protection against rabies infection in addition to contraception.

Suggestions for further Research

Further research should focus on discerning the molecular mechanisms involved in the developmental regulation of expression of ZP glycoproteins during folliculogenesis. The issue of presence of the ZPA and ZPC transcripts in granulosa cells in higher primates requires further confirmation. There is a need to further delineate the role of various zona pellucida glycoproteins in the complex cascade of events during fertilization in higher primates. The importance of carbohydrate moieties and their nature for recognition and binding of zona receptor on spermatozoa needs further investigations. The potential of recombinant ZP proteins/glycoproteins obtained by the use of various expression systems and synthetic peptides as immunogen for fertility regulation in different species should be investigated for its logical conclusion.

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