Zona pellucida glycoproteins based immunoccontraceptive vaccines: Strategies for development and their applications

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The mammalian oocyte is surrounded by an extra-cellular matrix, the zona pellucida (ZP), composed of three major glycoproteins (ZP1, ZP2 and ZP3). The ZP glycoproteins, by virtue of their tissue specificity and critical role during mammalian fertilization, have emerged as potential candidate antigens for the development of an immunoccontraceptive vaccine. Molecular characterization of ZP glycoproteins from several species, reveals a variable degree of homology among the deduced primary amino acid sequences, which provided an opportunity to undertake active immunization studies in heterologous animal models. Active immunization of various animal species with either native ZP glycoproteins or those obtained by recombinant DNA technology led to the inhibition of fertility. Thus ZP glycoproteins based immunoccontraceptive vaccines offer an attractive proposition for controlling wild life population. To make it a practical proposition, additional research inputs are required to optimize and devise novel strategies for vaccine delivery. Observed ovarian dysfunction, often associated with immunization by ZP glycoproteins is one of the major stumbling blocks for their use in humans. Ongoing studies to delineate appropriate B cell epitopes of ZP glycoproteins that are devoid of oophorogenic T-cell epitopes, which will inhibit fertility without concomitant oophoritis, will be critical to determine their feasibility for human use.

Keywords: Immunoccontraception, Ovary, Recombinant proteins, Synthetic peptides, Zona pellucida glycoproteins

It is projected that the global human population will be around ten billion by the year 2050. To control this increase in population, various contraceptives such as oral pills, intrauterine devices, condoms etc are available in addition to the terminal methods such as vasectomy for males and tubectomy for females. With an aim to provide a wider choice to end users, various groups across the world, are working on the development of immunoccontraceptive vaccines. This entails the generation of humoral and/or cell-mediated immune responses against antigens that have crucial role(s) in the process of reproduction. The proof of principle that it is feasible to design such a contraceptive vaccine has been demonstrated by extended phase-II clinical trials, of a vaccine based on the β-subunit of human chorionic gonadotrophin (β-hCG), in women. This vaccine was comprised of a heterospecies dimer of β-hCG annealed with the α-subunit of ovine lutetizing hormone (α-OLH) coupled to either tetanus toxoid (TT) or diphtheria toxoid (DT) as alternate carrier proteins. The vaccine was delivered with alum as an adjuvant. In addition, the first injection also contained a sodium phthalal derivative of lipopolysaccharides (SPLPS) as an additional adjuvant.

Immunization of women with this vaccine led to the generation of anti-hCG antibodies that neutralized the bioactivity of hCG. Immunized women having circulating neutralizing antibody titres above 50 ng/ml were protected against conception. Only one pregnancy was observed out of 1224 cycles1. However, an antibody titre above 50 ng/ml was observed only in 80% of the immunized women (n=148). Currently, efforts are ongoing to improve the immunogenicity of the β-hCG based vaccine by incorporating "promiscuous" T non-B cell epitopes instead of DT/TT as carrier proteins. Further, employment of adjuvants, permissible for human use, which are more potent than alum will also help in enhancing the immunogenicity of the vaccine.

The β-hCG based vaccine is aimed to interfere during implantation—a post-fertilization event. Another interesting target for the development of an immunoccontraceptive vaccine may be to interfere during fertilization per se. To achieve this, various groups have been engaged in the characterization of sperm antigens and their evaluation as potential candidate immunogens for the development of
immunocontraceptive vaccines. Alternately, it may be prudent to explore the potential of oocyte antigens for development of immunocontraceptive vaccines. The mammalian oocyte is covered by the zona pellucida (ZP), a thick, translucent, acellular glycoprotein matrix that plays an important role during fertilization. It serves as the “docking” site for the species-specific recognition and binding of the spermatozoa to the oocyte, induces acrosome-reaction in the zona-bound spermatozoa, affects avoidance of polyspermy and protects the pre-implantation blastocyst. ZP consists of three biochemically distinct glycoproteins, which have been classified as ZP1, ZP2 and ZP3 on the basis of their mobility on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Biochemical and structural aspects of ZP glycoproteins

In spite of having very similar polypeptide cores, differences in the post-translational modifications, including glycosylation leads to variability in the mobility of ZP glycoproteins isolated from various species on SDS-PAGE. In mice, under non-reducing conditions, ZP resolved as ZP1 (180-200 kDa), ZP2 (120-140 kDa) and ZP3 (83 kDa). Likewise, human ZP, under non-reducing conditions resolves into ZP1 (90-110 kDa), ZP2 (64-78 kDa) and ZP3 (57-73 kDa). The 2-D electrophoresis revealed that ZP glycoproteins are acidic in nature, and resolve as several isoelectric species, a result of differential glycosylation.

Analysis of genomic clones of ZP glycoproteins from various species have revealed similar genomic organization of a given gene family. The ZP1 family consists of 12 exons, ZP2 family 18 exons (human and cynomolgus monkey have an extra exon at the C-terminal end) and ZP3 family 8 exons. The 12 exons in mouse ZP1 (mZP1) range from 82-364 bp and encodes for a 623 amino acid (aa) polypeptide. The organization of exons in human ZP1 (hZP1) is similar to that in mZP1, spans 11 kb and encodes for a polypeptide of 540 aa. The exon size in mouse ZP2 (mZP2) ranges from 45-190 bp, with the transcript of 2201 nucleotide (nt) encoding for a 713 aa polypeptide while the human ZP2 (hZP2) has 19 exons transcribed into 2335 bp mRNA coding for a 745 aa polypeptide. Mouse ZP3 (mZP3) consists of 8 exons spanning 92-333 bp and encodes for a polypeptide of 424 aa. Human ZP3 (hZP3) also consists of 8 exons and encodes for a 424 aa polypeptide.

In addition to the genomic clones, the cDNA clones for the ZP glycoproteins from different species have been identified. The cDNA sequences have been characterized for mZP1, mZP2, mZP3, hamster ZP3, rabbit-55 kDa protein (homologue of mZP1), rabbit 75 kDa protein (homologue of mZP2), rabbit 45 kDa protein (homologue of mZP3), porcine ZP3α (homologue of mZP1), porcine ZP1 (homologue of mZP2) and porcine ZP3β (homologue of mZP3). In addition, the marmoset ZP3, hZP1, hZP2, hZP3, dog and cat ZP1, ZP2, ZP3, bonnet monkey ZP1 (bmZP1), ZP2 (bmZP2), and ZP3 (bmZP3) cDNA clones have also been characterized. The deduced primary aa sequences of ZP1, ZP2 and ZP3 from various species share a certain degree of sequence identity with the respective human counterparts as shown in Table 1. The comparison of the nt and deduced aa sequences of ZP glycoproteins from various species also revealed that they possess certain common features:

i) Short 5’ and 3’ untranslated regions.

ii) N-terminal signal peptide that directs the proteins into the secretory pathway and gets cleaved-off from the mature protein.

iii) Potential N- and O-linked glycosylation sites.

iv) C-terminal hydrophobic transmembrane-like domain that may play a role in the intracellular trafficking of the proteins.

v) A potential tetra basic furin cleavage site, upstream of the transmembrane-like domain.

vi) A ZP-signature domain.

Even though the ZP glycoproteins have been classified into three families, ZP1 and ZP2 from different species are more similar to each other as compared to ZP3. Comparison of the deduced aa sequence of bmZP1 Vs bmZP2 revealed a region from aa residues 369 to 418 of bmZP1, which has 52% identity with bmZP2. Within 348 aa of mZP1 (aa residues 268-623) that align with mZP2 (aa residues 363-713), 32% of the aa are identical. This region is encoded by 8 exons in both mZP1 and mZP2. This observation suggests that these 8 exons may have originated from the same ancestral gene that has been duplicated and re-utilized by exon shuffling. The conserved positions of 10 cysteine residues in mZP1 and mZP2 suggest that the structural aspects with respect to this domain may be similar in these two proteins. Further, the ZP domain, comprising of 253-260 aa with 8 conserved cysteines residues and additional conservation of hydrophobicity, polarity
and turn-forming tendency have been recognized in all the members of the ZP family except for cat ZP3 and mZPI. However, non-conformity of this domain in these two proteins is restricted to only one aa (aa residue 459 in mZPI and aa residue 204 in cat ZP3). In addition to members of the ZP family, the ZP domain is also present in extracellular matrix proteins such as the α- and β-tectorins of the inner ear, renal uromodulin (Tamm-Horsfall protein) and cuticulin. Other members of this family include membrane proteins, such as transforming growth factor-β-receptor type 3 (TGFB-III) and estrogen regulated gene type 1 (ERG1). Recently, it has been demonstrated that the ZP domain is responsible for polymerization of these proteins into filaments and assembly of ZP glycoproteins into the ZP matrices.

Evidence that ZP glycoprotein genes in mammals have diversified from other species such as fish during evolution has been accumulating. The primary structure of medaka (Orzizus latipes) egg vitelline envelope (VE) glycoprotein termed L-SF41 was found to be significantly similar to the ZP3 polypeptide. Moreover, 10 of the invariant cysteine residues have been conserved in this species suggesting an overall conservation of the three-dimensional structure of the ZP family through evolution. In flounders (Pseudopleuronectes americanus) the gene encoding another VE protein called wtfemale shares considerable sequence homology with the ZP2 protein family. Analysis of the sequence has revealed that exons 2-7 of this gene share homology with the rabbit ZP1 and mZPI. Furthermore, in the anuran (Xenopus laevis) the three cDNA cloned corresponding to the VE glycoproteins designated as gp37, gp41 and gp69 are homologues of the ZP glycoproteins ZP1, ZP3 and ZP2 respectively. Comparison of the deduced aa sequence of gp37 with the ZP1 sequence from human, porcine and mouse ZP1 revealed a sequence identity of 41.6%, 41.7% and 36.8% respectively. The gp69 revealed an aa sequence identity of 28.5%, 27.6% and 26.9% with mouse, pig and human ZP2. The gp41 showed an aa sequence identity of 40.9%, 40.0% and 40.8% with human, pig and mouse ZP3 respectively. Further analysis showed that they are not just related with respect to aa sequence homology observed with ZP glycoproteins from mammals but also had conserved positions of cysteine residues. Twenty out of 20 cysteine residues are conserved between gp37 and ZP1 from pig and human, nineteen in case of mZPI. All 12 cysteine residues were found to be conserved between gp41 and ZP3 from mice, pigs and humans.

**Functional aspects of ZP glycoproteins**

Studies from various experimental animal models have led to the elucidation of various functions associated with the ZP glycoproteins. In mice, hamster and humans, ZP3 acts as a primary receptor

<table>
<thead>
<tr>
<th>ZP Glycoprotein</th>
<th>Genome size/No. of exons</th>
<th>Apparent molecular wt (kDa)</th>
<th>Percent identity of deduced aa sequence with respective human homologue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ZP1 family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse ZP1 (mZP1)</td>
<td>6.5 kb/12</td>
<td>180-200</td>
<td>39</td>
</tr>
<tr>
<td>Pig ZP1</td>
<td>ND</td>
<td>~55</td>
<td>68</td>
</tr>
<tr>
<td>Bonnet monkey ZP1 (bmZP1)</td>
<td>ND</td>
<td>ND</td>
<td>92</td>
</tr>
<tr>
<td>Human ZP1(hZP1)</td>
<td>11 kb/12</td>
<td>90-110</td>
<td>100</td>
</tr>
<tr>
<td><strong>ZP2 family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse ZP2 (mZP2)</td>
<td>12.1 kb/18</td>
<td>120-140</td>
<td>60</td>
</tr>
<tr>
<td>Pig ZP2</td>
<td>25 kb/18</td>
<td>80-90</td>
<td>64</td>
</tr>
<tr>
<td>Bonnet monkey ZP2 (bmZP2)</td>
<td>ND</td>
<td>ND</td>
<td>94.2</td>
</tr>
<tr>
<td>Human ZP2 (hZP2)</td>
<td>13 kb/19</td>
<td>64-78</td>
<td>100</td>
</tr>
<tr>
<td><strong>ZP3 family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse ZP3 (mZP3).</td>
<td>8.6 kb/8</td>
<td>83</td>
<td>67</td>
</tr>
<tr>
<td>Pig ZP3</td>
<td>ND</td>
<td>~55</td>
<td>74</td>
</tr>
<tr>
<td>Bonnet monkey ZP3 (bmZP3)</td>
<td>ND</td>
<td>ND</td>
<td>93.9</td>
</tr>
<tr>
<td>Human ZP3 (hZP3)</td>
<td>18.3 kb/8</td>
<td>57-73</td>
<td>100</td>
</tr>
</tbody>
</table>
for sperm binding to the oocyte and induces the acrosome reaction in the spermatozoa bound to ZP. Following the acrosome reaction, in mice, ZP2 acts as a secondary sperm receptor and helps in the binding of the acrosome-reacted spermatozoa to the oocyte. Subsequent to fertilization, ZP2 undergoes limited proteolytic cleavage as a result of cortical reaction, resulting in the formation of small molecular weight fragments that remain non-covalently bound. In a mouse model, these changes accompanied by a loss of sperm receptor activity of ZP3 have been suggested to play an important role in preventing polyspermy. In the mouse model, ZP1 has been implicated to have a role in maintaining the structural integrity of the zona matrix by cross-linking ZP2-ZP3 heterodimer.

In the porcine model, high molecular weight hetero-complexes of ZP3 and ZP1 binds with a very high affinity to boar sperm-associated zona receptors whereas individual glycoproteins failed to do so thereby suggesting that both the glycoproteins are involved in sperm binding. In the rabbit model, rec55 (a homologue of mZPI, expressed in baculovirus) has been shown to bind to spermatozoa in a dose-dependent manner. Both rec45 (a homologue of mZPI3) and rec55 components of rabbit ZP have been shown to bind to recombinant Sp17 (sperm autoantigen), suggesting the involvement of a molecular mechanism similar to that found in the porcine system. Further, recombinant bmZPI (r-bmZPI) expressed in E. coli binds to the principal segment of the acrosomal cap of the capacitated spermatozoa and the equatorial segment, post-acrosomal domain and mid piece of the acrosome-reacted spermatozoa, indicating a role for ZPI in sperm binding.

Zona pellucida of mammalian oocyte as the target site for immuncontraception

The ZP glycoproteins, due to their critical role in the fertilization process, tissue specificity and accessibility to systemic antibodies have emerged as potential candidates for regulation of fertility through immunological intervention.

Active immunization with native ZP glycoproteins

(a) Total solubilized ZP

An early study demonstrated that immunization of mice with heat solubilized hamster zonae generated antibodies against ZP and induced infertility. Subsequently, porcine ZP became the antigen of choice due to its immunological cross-reactivity with human ZP and easy accessibility from abattoir. Immunization of rabbits with the heat solubilized porcine ZP generated immunological cross-reactive antibodies leading to infertility. The infertility was irreversible and histology of the ovaries revealed destruction of oocytes in all the growing follicles and severe depletion of the pool of resting follicles, suggesting that the infertility was due to ovarian dystrophy. Immunization of female dogs with porcine ZP resulted in prolonged pro-estrus or estrus cycles. Histological examination of ovaries from animals that generated high titres of antibodies against the immunogen revealed depletion of the oocytes. Immunization of non-human primates with crude solubilized porcine ZP also resulted in infertility that was irreversible and was accompanied by follicular atresia and abnormal hormonal profiles. The observed ovarian dystrophy following immunization with porcine ZP was attributed to the impurities present in the immunogens used.

(b) Purified ZP glycoproteins

To avoid the deleterious effects manifested due to the contaminating ovarian antigens, further studies were done using purified porcine ZP glycoproteins as the immunogens. Immunization of squirrel monkeys (Saimiri sciureus) with purified porcine ZP3 [a mixture of porcine ZP3α (-ZP1) and ZP3β (-ZP3)] induced disturbances in the secretion of ovarian steroid hormones initially and a histological examination of ovaries revealed interference in folliculogenesis. The immunized animals remained infertile during the study. In spite of high anti-porcine ZP3 antibody titres, the animals showed recovery of ovarian function after 10-15 months post-immunization. In another study, female bonnet monkeys (Macaca radiata) immunized with purified porcine ZP3, employing adjuvants permissible for human use also led to the inhibition of fertility. Immunized animals continued to have ovulatory cycles. Laproscopic examination revealed normal ovaries with developing follicles. Following the decline in antibody titres, 50% of the animals became pregnant. Ovarian histology of the animals that failed to regain fertility did not reveal any signs of inflammation or lymphocytic proliferation. There was also no observed increase in the number of atretic or degenerating follicles. These studies suggest that some of the adverse effects on ovarian functions can
be minimized subsequent to immunization with the purified ZP glycoproteins.

In addition to the contaminating proteins in ZP glycoproteins, the nature of the adjuvants employed in active immunization studies will also influence the efficacy as well as the safety of the procedure. Use of complete Freund's adjuvant (CFA) leads to very high antibody titres but is also accompanied by granulomatous lesions at the site of injection and ovarian atrophy. Use of adjuvants such as aluminium hydroxide gel (alum), sodium phthalyl derivative of lipopolysaccharides (SPLPS) and synthetic muramyl dipeptide derivative (MDP) also generates an immune response to ZP antigens, capable of blocking fertility with less adverse side effects. Hence, there is a need to develop more potent and safer adjuvants.

Active immunization with recombinant ZP proteins/glycoproteins

Contamination of the ZP based antigens by other ovary associated proteins, one of the causes for ovarian pathology, has been circumvented by the use of recombinant ZP proteins/glycoproteins. Immunization of marmosets (Callithrix jacchus) with the recombinant hZP3 (r-hZP3) expressed in CHO mammalian cells generated antibodies that led to long-term infertility. However, this infertility was associated with ovarian pathology characterized by depletion of primordial follicles. Immunization of female rabbits with recombinant rabbit ZP1, expressed in eukaryotic cells, caused infertility in 70% of the immunized animals. In the same study, female rabbits were also immunized with recombinant myxoma virus harboring the ZP1 gene followed by boosting with recombinant ZP1, which resulted in 80% infertility. The infertility was associated with follicular degeneration and depletion of primordial follicles. In another study, a comparative active immunization of female cynomolgus monkey (Macaca fascicularis) and baboons (Papio cynocephalus) with purified r-hZP1, r-hZP2 and r-hZP3 expressed in CHO cells revealed that those animals which were immunized with r-hZP2 or r-hZP3 became pregnant before any of the animals immunized with r-hZP1. The animals immunized with r-hZP1 remained infertile for 9-35 months. During the time of high antibody titres, some animals experienced disruption of the menstrual cycle, which eventually returned to normal. These studies demonstrate that ZP1 is a better candidate for fertility regulation as compared to ZP2 or ZP3. Our group has also shown that immunization of female baboons (Papio anubis) with recombinant bmZP1 (r-bmZP1) conjugated to DT (r-bmZP1-DT) generated high antibody titres against r-bmZP1. The immunized animals exhibited normal ovulatory cycles. In the presence of high antibody titres, they remained infertile following mating with males of proven fertility. However, subsequent to the decline in antibody titres the animals conceived. In addition, using a homologous animal model, female bonnet monkeys were also immunized with r-bmZP1-DT and r-bmZP2-DT conjugates. Immunization led to the generation of antibodies against both the respective recombinant proteins as well as DT. The immunized females remained infertile when mated with males of proven fertility in the presence of high antibody titres.

Table 2 — Effect of immunization with recombinant zona pellucida proteins on fertility in female bonnet monkeys

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of animals used</th>
<th>Status of fertility</th>
<th>Ovarian histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>Two females conceived in first ovulatory cycle and remaining 2 in the second ovulatory cycle.</td>
<td>Normal follicular development</td>
</tr>
<tr>
<td>Immunized with r-bmZP1-DT</td>
<td>5</td>
<td>Cumulative infertility observed for 45 ovulatory cycles. One animal conceived during immunization when antibody titres were low and another conceived during reversal studies. Three animals failed to conceive even after the decline of antibody titres to background.</td>
<td>Folliculogenesis disturbed. Atretic follicles showed degenerated oocytes with disorganized ZP</td>
</tr>
<tr>
<td>Immunized with r-bmZP2-DT</td>
<td>4</td>
<td>Cumulative infertility observed for 32 ovulatory cycles. One animal conceived during immunization when antibody titres were low and another conceived during reversal studies. Two animals failed to conceive even after the decline of antibody titres to background.</td>
<td>Same as with r-bmZP1-DT immunized group</td>
</tr>
</tbody>
</table>

*Adapted from Govind et al.*
and failed to conceive even after the decline of the anti-r-bmZP1 and anti-r-bmZP2 antibody titres (Table 2). Ovarian histopathology of the immunized animals revealed the presence of atretic follicles and degenerated oocytes, which may have been the principle cause for the block in fertility.

Various recombinant fragments of mZP2 have also been evaluated, in a homologous animal model, for their efficacy to block fertility. These studies revealed that immunization with the mZP2 fragment corresponding to aa residues 35-637 led to a reduction in fertility. The ovaries of the infertile mice were histologically normal and produced a normal number of eggs suggesting that the mouse ZP2 antibodies probably induce infertility by interfering with sperm-oocyte interactions. This study also demonstrated the efficacy of a chimeric recombinant protein comprising of mZP2 and sperm antigen, Sp17, to inhibit fertility.

Alternate modes of immunization

Along with conventional immunization, various other modes and routes of delivery of ZP glycoproteins have been tried to generate more effective immunocontraceptive vaccines.

(a) Live recombinant vectors

Oral immunization of female mice with recombinant Salmonella expressing mZP3 induced significant levels of IgG type of antibodies against ZP in the serum. In addition, significant levels of IgA antibodies against ZP were also observed in the vaginal secretion. Infertility was observed in 3 out of 6 immunized mice. In another study, ectomelia virus – a natural pathogen for mice that causes mouse pox, has been used as a live vector. A recombinant ectomelia virus expressing mZP3 was made. Immunization of a group of mice with recombinant viruses led to a decrease in fertility as well as litter size compared to the group immunized with viruses containing only the plasmid. Immunization led to disruption of follicular development but did not cause any ovarian oophoritis.

(b) DNA vaccines

Another alternative approach to conventional immunization with protein is to use plasmid DNA encoding the said protein. The feasibility of this concept was demonstrated for the first time by generating protective immunity against influenza subsequent to immunization with a DNA vaccine. Since then, numerous studies have shown that immunization with plasmid DNA encoding a variety of antigens corresponding to a wide spectrum of bacteria, viruses and protozoa leads to generation of protective humoral and/or cell-mediated immune responses. As compared to conventional vaccines, DNA vaccines have several advantages. They are easier to produce and hence cheaper. Plasmid DNA is amenable to easy manipulation and hence DNA vaccines can be modified with ease. They are quite stable at room temperature and may not require a cold chain. The injected DNA mostly remains in the form of an episome, thus avoiding the fear of integration into the host genome. Although the immune response to DNA vaccines is weak as compared to that induced by traditional vaccines, it is exceptionally long-lasting.

Our group has recently evaluated the potential of DNA vaccines with respect to immunocontraceptive vaccines. In this direction, the cDNA corresponding to bmZP1, excluding the N-terminal signal sequence and C-terminal transmembrane-like domain, was cloned in the mammalian expression vector VR1020, downstream of a tissue plasminogen activator signal sequence under a cytomegalovirus promoter. Immunization of male mice with the above plasmid DNA vaccine in saline led to generation of the antibodies against E. coli expressed r-bmZP1. Administration of r-bmZP1 in mice immunized with a DNA vaccine encoding bmZP1, led to a further increase in the antibody titres. The antibodies thus generated recognized the native ZP both from bonnet monkeys as well as humans. Interestingly, the immune sera obtained from mice immunized with the DNA vaccine showed significant in vitro inhibition of the binding of spermatozoa to ZP in the hemizona assay as compared to the immune sera obtained from mice immunized with the vector. Similarly, a DNA vaccine encoding dZP3 also elicited an antibody response that recognized the native dog ZP. A dominant IgG1 isotype response was observed in animals immunized with the DNA vaccine using a gene gun as compared to a mixed IgG1-IgG2a isotype response when delivered in saline. Interestingly, female mice immunized with a DNA vaccine using a gene gun also generated antibodies that recognized r-dZP3, as well as native dog and bonnet monkey ZP. Keeping this in view, it may be of interest to undertake active immunization studies of female dogs with the DNA vaccine encoding dZP3 to see its effect on fertility.
Relevance of ZP glycoproteins in controlling wild life population

Understanding the molecular basis of fertilization and relevance of ZP glycoproteins in this process will have direct bearing on devising strategies to conserve several species that are on the verge of extinction. At the same time, an unchecked increase in the population of some species such as deer and elephants in forests and street dogs has been observed. New legislations in many countries, which forbid hunting and killing of these animals, have further aggravated the situation. Hence, there is a need to develop new strategies to control an increase in wild life population in a more humane way. An immunococeptive vaccine for humans should be effective in 100% of the recipients, should be potentially reversible, and free of side effects. For wild life control, a vaccine leading to an irreversible block in fertility may also be acceptable, and in some situations desirable, as it will be akin to an immunological castration. Further, such a vaccine meant for female animals and having an efficacy of about 50-70% will also be effective in regulating wild life population.

In feral horse and donkey populations, a single annual booster of solubilized porcine ZP was enough to prevent conception, without affecting the complex social behavior of the animals. Short-term treatment for up to 4 consecutive years did not result in any detectable debilitating side-effects and the contraceptives effect was reversible while long-term treatment (5-7 years) was associated with few ovulation failures and depressed urinary estrogen levels.

Out of the 74 species of captive zoo animals immunized with solubilized porcine ZP, 27 species have shown distinct successes in block of fertility. Immunization of white-tailed deer (Odocoileus virginianus) with either native porcine solubilized ZP or recombinant rabbit ZP glycoproteins led to a significant decrease in the fawning rates. In a field trial carried out for control of elephant population, it was demonstrated that out of 19 female elephants vaccinated with porcine ZP, 10 did not conceive. However, in the control group, 16 out of 18 animals became pregnant. The effects of the vaccine were reversible, and had no deleterious effects on the ovary and its cyclicity. A single shot of porcine solubilized ZP using a liposome delivery system decreased the birth of pups in the gray seals (Halichoerus grypus) by 90% over a five-year period.

The potential of ZP glycoproteins as candidate antigens have also been studied to inhibit fertility in dogs. For this purpose, the cDNA encoding dog ZP2 (dZP2) and dog ZP3 (dZP3), excluding the N-terminus was used to express recombinant ZP proteins in E. coli. The upper panel is a schematic representation of the construct of dZP3 in pQE30 expression vector. The SGI3009[pREP4] E. coli cells were used for the expression of the recombinant protein as polyhistidine fusion protein. The expressed r-dZP3 was purified on a nickel-nitrotriacetic acid agarose affinity column. The lower panel represents the SDS-PAGE profile of the purified r-dZP3 stained with Coomassie blue. P7, promoter of phage T7; Lane M, molecular weight markers; Lane 1, purified r-dZP3.

![Fig. 1 — Expression of recombinant dog ZP3 in E. coli. The upper panel is a schematic representation of the construct of dZP3 in pQE30 expression vector. The SGI3009[pREP4] E. coli cells were used for the expression of the recombinant protein as polyhistidine fusion protein. The expressed r-dZP3 was purified on a nickel-nitrotriacetic acid agarose affinity column. The lower panel represents the SDS-PAGE profile of the purified r-dZP3 stained with Coomassie blue. P7, promoter of phage T7; Lane M, molecular weight markers; Lane 1, purified r-dZP3.](https://example.com/fig1)

**Table 3 — Effect of immunization with dog recombinant zona pellucida proteins on fertility in female dogs**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of animals used</th>
<th>Status of fertility</th>
<th>Ovarian histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunized with DT</td>
<td>4</td>
<td>Out of 4 immunized animals, three became pregnant on mating during estrus</td>
<td>Normal folliculogenesis with healthy oocytes</td>
</tr>
<tr>
<td>Immunized with r-dZP2-DT</td>
<td>4</td>
<td>All the four immunized female dogs conceived on mating during estrus</td>
<td>Normal folliculogenesis</td>
</tr>
<tr>
<td>Immunized with r-dZP3-DT</td>
<td>4</td>
<td>Out of four immunized animals, three failed to conceive when mated during estrus</td>
<td>Atretic follicles, degenerative changes in ZP</td>
</tr>
</tbody>
</table>

*Adapted from Srivastava et al. 5*
terminal signal sequence and the C-terminal transmembrane-like domain, were cloned in pQE-30 expression vector under T7 promoter. The respective protein was expressed as a polyhistidine fusion protein in E. coli. The vector map and SDS-PAGE profile of the purified r-dZP3 is shown in Figure 1. The purified r-dZP3 and r-dZP2 were conjugated to DT. The female dogs were immunized with r-dZP2-DT and r-dZP3-DT conjugates. Immunization led to the generation of antibody response against the respective ZP proteins as well as the carrier. All the female dogs immunized with r-dZP2-DT conceived subsequent to mating (Table 3). Three out of four animals immunized with DT also conceived. However, 3 out of 4 animals immunized with r-dZP3-DT remained infertile (Table 3). Ovarian histopathology of the animals immunized with r-dZP3-DT revealed follicular atresia. These preliminary results indicate that the dog population can be controlled by immunization with r-dZP3, provided adequate antibody titer are achieved.

**Prospects of developing a ZP glycoprotein based immuno-contraceptive vaccine for human use**

As mentioned earlier, it is imperative that ZP glycoproteins based contraceptive vaccines for humans should not have any side effects. The presence of oophorogenic T cell epitope(s) in the ZP glycoproteins based immunogens has also been implicated as one of the factors responsible for the observed changes in sex-steroid hormones profile, disturbance in cyclicity and disrupted ovarian follicle development, subsequent to immunization with ZP glycoproteins. Hence, several groups are engaged in finding the minimum effective peptide of the three ZP glycoproteins that could produce fertilization-blocking antibodies without undesirable side effects. The first evidence to show its feasibility was demonstrated by synthesizing a chimeric peptide comprising of a “promiscuous” T-cell epitope of bovine RNase (NCAYKITQANK), co-linearly synthesized with the minimal B-cell epitope of mZP3 corresponding to aa residues 335-342, with phenylalanine aa replaced by alanine (QAQHGPR) (Table 4). The peptide elicited antibodies in inbred mice of eight different haplotypes without activation of oophorogenic T cells. When mated, the litter size of the immunized group was considerably lower as compared to that of the control group. Moreover, the reduction in the litter size correlated with the antibody titers. For the first time, these experiments showed a method of overcoming the MHC driven non-responsiveness to a self-antigen and also avoiding self-pathogenic T cell responses. In analogy, cynomolgus monkeys were immunized with a chimeric peptide comprising of *Plasmodium falciparum* T cell helper epitope (aa residues 326-337) and bZP3 (aa residues 334-342; RRQPHVMS). Antibodies thus elicited, significantly inhibited the *in vitro* binding of human sperm to antibody treated zona encased oocytes as compared to pre-immune sera. Results from our group also showed that immunization of female mice with a chimeric peptide corresponding to bmZP3 (aa residues 334-343) synthesized co-linearly with a “promiscuous” T cell epitope of circumsporozoite protein (CSP, aa residues 378-398) of *Plasmodium falciparum* elicited antibodies that inhibited, *in vitro*, sperm-zona binding.

As described above, immunization of female marmosets with r-hZP3 and of female dogs with r-dZP3-DT led to infertility concomitant with ovarian dysfunction. Immunization of female marmosets with recombinant marmoset ZP3 also leads to a block in fertility associated with disturbances in ovarian follicular development. In contrast, no disruption of ovarian function was observed following immunization of marmosets with synthetic peptides comprising of a “promiscuous” T-cell epitope of bovine RNase (NCAYKITQANK), co-linearly synthesized with the minimal B-cell epitope of mZP3 corresponding to aa residues 335-342, with phenylalanine aa replaced by alanine (QAQHGPR) (Table 4). The peptide elicited antibodies in inbred mice of eight different haplotypes without activation of oophorogenic T cells. When mated, the litter size of the immunized group was considerably lower as compared to that of the control group. Moreover, the reduction in the litter size correlated with the antibody titers. For the first time, these experiments showed a method of overcoming the MHC driven non-responsiveness to a self-antigen and also avoiding self-pathogenic T cell responses. In analogy, cynomolgus monkeys were immunized with a chimeric peptide comprising of *Plasmodium falciparum* T cell helper epitope (aa residues 326-337) and bZP3 (aa residues 334-342; RRQPHVMS). Antibodies thus elicited, significantly inhibited the *in vitro* binding of human sperm to antibody treated zona encased oocytes as compared to pre-immune sera. Results from our group also showed that immunization of female mice with a chimeric peptide corresponding to bmZP3 (aa residues 334-343) synthesized co-linearly with a “promiscuous” T cell epitope of circumsporozoite protein (CSP, aa residues 378-398) of *Plasmodium falciparum* elicited antibodies that inhibited, *in vitro*, sperm-zona binding.

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**Table 4** — Zona pellucida glycoprotein-3 based synthetic peptide immunogens

<table>
<thead>
<tr>
<th>Synthetic peptide</th>
<th>Animal species</th>
<th>Outcome of active immunization</th>
<th>Reference number</th>
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<tbody>
<tr>
<td>mZP&lt;sub&gt;335-342&lt;/sub&gt;</td>
<td>Mice</td>
<td>Block in fertility without concomitant oophoritis</td>
<td>53</td>
</tr>
<tr>
<td>Ph&lt;sub&gt;335&lt;/sub&gt; replaced by Ala</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marmoset ZP3&lt;sub&gt;334-342&lt;/sub&gt;</td>
<td>Marmoset</td>
<td>Normal ovarian functions. Antibodies against peptide showed <em>in vitro</em> contraceptive efficacy.</td>
<td>56</td>
</tr>
<tr>
<td>bmZP&lt;sub&gt;326-347&lt;/sub&gt;</td>
<td>Bonnet monkey</td>
<td>Block in fertility. No disruption of cyclicity. Normal folliculogenesis.</td>
<td>57</td>
</tr>
</tbody>
</table>
corresponding to human or marmoset ZP3\(^{35,56}\). Antibodies against the marmoset ZP3 peptide, corresponding to aa residues 301-320, recognize both marmoset and human native ZP and also reduced in vitro human sperm-zona interaction by 60% (Table 4). However, the in vivo studies did not show consistent reduction in fertility\(^{36}\). Immunization of female bonnet monkeys with a synthetic peptide corresponding to bmZP3 (aa residues 324-347) conjugated to DT led to high antibody titres against the peptide\(^{55}\). Immunized animals continued to have ovulatory cycles (except summer amenorrhoea) and failed to conceive when mated with males of proven fertility. No ovarian pathology was observed in the immunized animals (Table 4).

To enhance the immunoc ontraceptive efficacy of anti-peptide antibodies, it may be prudent to design synthetic chimeric immunogens encompassing multiple epitopes of a given zona glycoprotein or multiple zona glycoproteins. The above premise is derived from the observations that a cocktail of anti-peptide antibodies against porcine ZP3\(^{-}\) (–ZP3) was effective in inhibiting porcine sperm-oocyte attachment in vitro whereas individual anti-peptide antibodies failed to do so\(^{55}\). On the other hand, female bonnet monkeys immunized with a physical cocktail of bmZP3 peptide, individually conjugated to DT, generated antibodies that significantly inhibited human sperm-oocyte binding in vitro\(^{55}\). This was probably mediated by a cooperative effect among the antibodies pertaining to different domains. Taking a cue from these observations, we have recently cloned and expressed a chimeric recombinant protein encompassing B cell epitopes of bmZP1 (aa residues 132-147), bmZP2 (aa residues 86-113) and bmZP3 (aa residues 324-347) in E. coli\(^{40}\). Female rabbits immunized with the chimeric recombinant protein conjugated to DT generated antibodies that not only reacted with the respective recombinant proteins but also with the bonnet monkey and human native ZP. The immune sera also inhibited the in vitro binding of human spermatozoa to the human zona in a hemizona assay. In another study, female marmosets were immunized with a triple peptide vaccine encompassing marmoset ZP3 epitopes (aa residues 85-100, 241-260 and 301-320) including a “promiscuous” T-helper cell epitope\(^{61}\). Immunization of animals elicited high antibody titres against regions corresponding to aa residues 241-260 and 301-320. The third peptide from aa residues 85-100 was poorly immunogenic. Immune sera showed in vitro contraceptive efficacy. No loss of ovarian function was observed in the immunized animals.

Synthetic peptides corresponding to ZP2 have also been targeted for the purpose of immun contraception. It was shown that rabbit polyclonal antibodies against hZP2 peptide corresponding to aa residues 541-555 inhibited in vitro human sperm binding to the human ZP by about 50\(^{62}\). Immunization of mice with a chimeric peptide comprising of mZP2 epitope (aa residues 121-140) and bovine RNase “promiscuous” T-cell epitope reacted with native mouse ZP and did not lead to oophoritis\(^{63}\). Rabbits immunized with an 18-mer synthetic peptide corresponding to hZP2 (aa residues 50-67) conjugated to DT not only recognized the native human ZP but also effectively inhibited the in vitro binding of human sperm to human ZP\(^{55,65}\).

To map the B-cell epitope on ZP1, we have employed two approaches. In the first approach, monoclonal antibodies were generated against r-bmZP1\(^{16}\). Those monoclonal antibodies, which were capable of recognizing the native human ZP and had a significant inhibitory effect on the in vitro binding of human sperm to human zona, were selected. Epitope mapping studies, using these monoclonal antibodies, revealed a common epitope, DAPDTDWCDSIP (aa residues 136-147). In the second approach, 4 peptides corresponding to bmZP1 were synthesized on the basis of computational predictions of hydrophilicity, surface probability and antigenicity\(^{53}\). Female mice were immunized with the synthetic peptides corresponding to bmZP1, conjugated individually with DT. Characterization of the immune serum samples revealed that antibodies against the bmZP1 peptide corresponding to aa residues 251-273 inhibited the binding of human spermatozoa to ZP in a hemizona assay\(^{55}\). These studies will help in the identification and characterization of synthetic peptide immunogens that may be devoid of oophorogenic T-cell epitopes and ultimately lead to the design of immunogens encompassing B-cell epitopes for better efficacy in inhibiting fertility.

**Concluding comments**

The findings that immunization with either native or recombinant ZP proteins/glycoproteins inhibits fertility, in several species, may find application in controlling wild life population. Novel strategies, such as DNA vaccine or live vectors encoding ZP glycoproteins need further evaluation. To make the use of such vaccines a practical proposition, alternate routes of vaccine delivery, such as orally or by using
Darts have to be evolved. It may also be desirable to reduce the number of vaccine inoculations. In order to develop a safer and effective ZP glycoprotein based immuncontraceptive vaccine for human use, it is imperative to have a comprehensive understanding of the mechanisms involved in gamete interaction. Thus, efforts to delineate new immunogens, comprising of B-cell epitopes of ZP glycoproteins and devoid of oophoritogenic T cell epitopes must continue.

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