—Review—

Evaluation of the Contraceptive Potential of Recombinant Proteins and Synthetic Peptides of Zona Pellucida (ZP)

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The increase in the global human population necessitates fertility control systems that have high efficacy, are free from side effects and are reversibile. Extensive research has been conducted on the development of contraceptive vaccines using specific gamete antigens. One target for immunocontraception is the zona pellucida (ZP), the extracellular matrix surrounding growing oocytes, ovulated eggs and preimplantation embryos. The ZP is believed to be a promising candidate because of its important role at fertilization [1], its tissue-specific expression [2, 3] and its strong immunogenicity [4, 5].

Early studies showed that the ZP possesses strong immunogenicity against heterologous species with antisera effectively blocking fertilization [4–7]. Immunized animals produced antibodies reactive to their own ZP and became infertile. On the basis of this species cross-reactivity of zona antigens [8–11], intensive investigations have been conducted on the development of contraceptive vaccines [12–15], but the animals immunized with ZP antigens were also subject to ovarian dysfunction and depletion of primordial follicles resulting in premature menopause [16–18]. Therefore, it is necessary to segregate the epitope that inhibits the sperm-ZP interaction from other epitopes that induce ovarian failure.

Biochemical and immunological analyses have revealed that the mammalian ZP is composed of three or four major glycoproteins [19–23]. In mice three ZP glycoproteins have been identified: ZP1, ZP2 and ZP3. This classification is based on apparent molecular masses as determined by one-dimensional SDS-PAGE [19]. After DNA cloning of each glycoprotein, it was found that ZP1, ZP2 and ZP3 were encoded by the genes *zpB*, *zpA* and *zpC*, respectively. This designation is based on decreasing length of the relevant messenger RNAs, *zpA* mRNA being the longest [24]. In pigs, two-dimensional electrophoresis (isoelectric focusing and molecular sieving) showed that the ZP was composed of four glycoprotein families with high pI heterogeniety [25, 26]. This is probably due to the heterogeneous nature of the carbohydrate chains. The four glycoprotein families were designated as pig ZP1, ZP2, ZP3 and ZP4 in order of decreasing molecular weight. Further analysis showed that pig ZP2 and ZP4 were derived from parental ZP1 [26, 27], and that the pig ZP3 family included two distinct core proteins, ZP3α and ZP3β, [28]. The pig ZP genetically comprised three glycoproteins of pig ZP1, ZP3α and ZP3β. The DNA cloning of pig ZP glycoproteins revealed that pig ZP1, ZP3α and ZP3β are homologues of the products of *zpA*, *zpB* and *zpC*, respectively. The terminology of the ZP glycoproteins can be rather confusing since different names are used for different animal species before DNA cloning. Evidence now indicates that ZP glycoproteins in different species are derived from the common ancestor of genes *zpA*, *zpB* and *zpC*. In this article the three ZP glycoproteins are referred to as ZPA, ZPB and ZPC based on the designation of the relevant genes (Table 1).

Recent advances in recombinant DNA technology and protein engineering are making the development of contraceptive vaccines a reality [15, 29–31]. This is now possible because once the amino acid sequence of a protein is identified, the specific epitopes or the minimum region required for the vaccine targets can be determined. This article describes the current status and potential of contraceptive vaccine development based on ZP antigen.

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Table 1. Designation of zona pellucida glycoproteins from various species

ZP* protein	pig	mouse	human	rabbit
ZPA	ZP1 (ZP2 + ZP4)	ZP2	ZP2	R75K
ZPB	ZP3α	ZP1	ZP1	R55K
ZPC	ZP3β	ZP3	ZP3	–

*ZP: Zona Pellucida.

Recombinant Vaccines

1) Current status

In previous studies, several recombinant ZP proteins were used for immunization of mammals, including rabbit ZPA, ZPB [32–34], monkey ZPA, ZPB, ZPC [35–37] and human ZPC [31].

The recombinant rabbit ZPA and ZPB produced in *E. coli.* failed to induce antibodies in cynomolgous monkeys [32, 33], but when they were coupled to carrier proteins, both immunogens could induce antibodies. The animals immunized with ZPA elicited ovarian failure, whereas the aminals immunized with ZPB did not. The serum from the latter animals inhibited sperm binding to the ZP in cynomolgous monkeys. The recombinant rabbit ZPB produced in insect cells was immunogenic without carrier proteins in rabbits and guinea-pigs, and the antisera reacted with native ZP of rabbits [34]. Glycosylation by the insect cells seemed to enhance the immunogenicity of the recombinant rabbit ZPC protein. Unfortunatly, the effect of the antisera on fertilization is unknown.

Intensive studies were also carried out on bonnet monkey ZP proteins. Isolated cDNAs coding for ZPA, ZPB and ZPC were used for producing recombinant proteins in a bacterial system [35–37]. The recombinant ZPB and ZPC proteins induced antibodies which reacted with native ZP from bonnet monkeys only when conjugated to a carrier protein [36, 37]. In contrast, the recombinant ZPA induced antibodies without a carrier protein and the antisera raised in rabbits reacted to native ZP from bonnet monkeys and humans [35]. The amino acid sequences of bonnet monkey ZPA, ZPB and ZPC were very similar to those of humans (94.2%, 92.0% and 93.9%, respectively), indicating that active immunization of bonnet monkeys would be a good experimental model for humans.

A study on recombinant human ZPC was reported by Aitken's group. They developed a method for large scale production of soluble recombinant human ZPC glycoprotein in Chinese hamster ovary cultured cells [38]. The recombinant human ZPC glycoprotein produced antibodies in marmosets without any carrier protein. Glycosylation may have enhanced the immunogenicity of recombinant human ZPC proteins as was the case with rabbit recombinant ZPC glycoprotein. The sequence homology of marmoset and human ZPC is 91%. The marmosets which were immunized with recombinant human ZPC had varyious degrees of ovarian failure.

Collectively, both glycosylated and non-glycosylated recombinant ZP proteins seem to contain B cell epitopes which could induce antibodies reactive to native ZP, but ovarian failure also occurred in the immunized animals. Therefore, it is necessary to identify and characterize B cell epitopes of the recombinant ZP proteins which induce fertilization blocking antibodies without any ovarian dysfunction.

2) Antiserum to recombinant pig ZPA (r-pZPA)

Our previous studies showed that the NH_2-terminal region of pig ZPA, corresponding to pig ZP4, was strongly immunogenic and the antisera raised in mice could block human *in vitro* fertilization (IVF) [39]. Recently we succeeded in cloning the cDNA and genomic DNA of pig ZPA [40] and constructed an expression vector (pET-21b) of cDNA coding for the NH_2-terminal region of pig ZPA (198 amino acids excluding the signal sequence of 35 amino acids). The recombinant protein, r-pZPA (1-198), was produced at a high concentration, 100 μg/ml of culture broth by *E. coli.* Nickel immobilized resin was used for purification since r-pZPA (1-198) was expressed as a fusion protein with polyhistidines (6X). Immunization in rabbits was carried out by intradermal injection with 200 μg of r-pZPA emulsified in complete Freund's adjuvant. The animals received 3 monthly booster injections with the same amount of the protein in incomplete Freund's adjuvant. The immunization of r-pZPA (1-198) induced high titer antibodies in rabbits without any carrier proteins. The generated antibodies reacted not only with pig ZP but also with human ZP, suggesting interspecies cross-reactivity between pigs

and humans in this region of r-pZPA. When the antiserum was added to a medium of pig IVF at a concentration of 10%, the fertilization rate was significantly decreased (Table 2), but the antiserum did not inhibit human IVF. One possible reason is that the antiserum to pigs did not induce high avidity/affinity antibodies to human ZP. Therefore, we attempted to produce antibodies that block human IVF by using a recombinant human ZP protein.

3) Antiserum to recombinant human ZPA (r-hZPA)

cDNA coding for human ZPA was provided by Dr. Jurrien Dean (National Institute of Health, USA). The cDNA coding for the NH_2-terminal region (206 amino acids) was excised by EcoR1 and inserted into an expression vector (pET21b). The cDNA fragment corresponded to r-pZPA (1-198) with the addition of 8 amino acids at the COOH terminal end; it did not contain the signal peptide sequence (38 amino acids). The yield of the recombinant human ZPA, r-hZPA (1-206), caused by *E. coli* was similar to that of r-pZPA (1-198), 100 μg/ml culture broth. Purification and immmunization were carried out in the same way as for r-pZPA (1-198). r-hZPA also induced high titer antibodies to r-hZPA (1-206) and cross-reacted with r-pZPA. In immunofluorescent staining, the anti r-hZPA antiserum reacted with native ZP from pigs and humans, as did the antiserum to r-pZPA. When 10% antiserum was added to human IVF medium, the numbers of sperm bound to and penetrating the ZP were significantly reduced, as shown in Table 3. These results clearly demonstrated that antiserum to homologous recombinant protein was more effective in blocking human IVF.

Peptide Vaccines

1) Current status

The first study for the determination of a linear B-cell epitope was reported by Millar *et al.* [23]. The epitope was localized to the 336–342 peptide of mouse ZPC. They synthesized a 16 mer peptide (327–342) and conjugated it to a keyhole limpet haemocyanin (KLH) as a carrier protein for immunization. Immunized female mice subsequently developed long-term infertility with concomitant oophoritis. Lou and Tung [41] were able to show that the oophoritis was caused by a T-cell epitope included in the 16 mer peptide.

Human and monkey synthetic peptides corresponding to the mouse ZPC peptide were prepared to test immunogenicity and contraceptive effects. Gupta *et al.* [42] investigated the immunogenicity of a 19 mer peptide (323–341) from human ZPC with an additional 4 amino acids at the NH_2-terminal end. This peptide, conjugated to diphtheria toxoid (DT), elicited antibodies reactive to bonnet monkey and human ZP in hamsters.

Mahi-Brown and Moran also synthesized a human ZPC peptide (327–341) and conjugated it to two different carrier proteins for immunization in mice [43]. They showed that the raised antibodies recognized human and macaque ZP but not canine ZP. In their next report, they prepared several formulations for homologous monkey immunization with a cynomolgus ZPC peptide [29]. The peptide conjugated to tetanus toxoid (TT) produced antibodies to the immunogen with the highest titer, but it was not reported whether the antibodies re-

Table 2. Effect of addition of antiserum to r-pZPA on pig *in vitro* fertilization

Antisera	No. of eggs used	No. of eggs fertilized**	%	
NRS*	37	33	89.2	***
Anti r-pZPA	39	6	15.4	

*NRS: Normal rabbit serum. **Enlarged sperm heads were observed in cytoplasm in fertilized eggs. ***$p<0.01$ (χ^2 test).

Table 3. Effect of addition of antiserum to r-hZPA on human *in vitro* fertilization

Antisera	No. of eggs used	No. of sperm bound to ZP/egg (Mean ± S.D.)		No. of sperm penetrating into ZP/egg (Mean ± S.D.)
NRS*	9	75.1 ± 23.3	**	3.4 ± 2.2
r-hZPA	8	5.8 ± 5.1		0 ± 0

*NRS: Normal rabbit serum. **$p<0.01$ (t test).

Table 4. Characteristic properties of MAb-5H4

IgG subclass	IgG2b (mouse)
Recognization molecule	ZPA
Species cross-reactivity	Pig, Human, Rabbit, Dog, Cat, Bovine
Tissue specificity	Zona pellucida
Biological function	Blocking of fertilization
Reactive synthetic peptide	CTYLDPENLTLKAPYEA (50–67)
Minimum binding motif	LDPENLTL (54–61)

acted with native ZP or not.

A number of synthetic peptides of human ZPC (45–64, 93–110, 172–190, 341–360) were tested for their immunogenicity [31]. They were selected based on their reactivity with rabbit antiserum produced to native human ZP. A series of 8mer peptides, offset by one amino acid on each successive peptide were synthesized onto polyethylene pins covering the whole human ZPC. They found that two peptides (45–64, 172–190) conjugated to TT produced antibodies in marmosets, but the reactivity to marmoset ZP was very weak. Bagavant *et al.* reported that a chimeric peptide of human ZPC peptide (334–342) and a foreign T cell epitope produced specific antibodies to ZP from cynomolgous monkeys and that some antisera inhibited human sperm binding to the ZP [30].

In pig synthetic peptides, the NH_2-terminal portion of pig ZPB and ZPC was reported to be immunogenic when conjugated to KLH [44]. Here it was shown that only the antiserum to the peptide of ZPC inhibited boar sperm binding to the ZP. Five synthetic peptides of pig ZPB were examined to determine whether or not they produced antibodies cross-reactive with human ZP. Two of the peptides recognized native ZP on marmoset ovarian section. These peptide sequences were determined by hydropathicity analysis [45].

2) Epitope for a monoclonal antibody (MAb-5H4)

With pig ZPA we were able to produce several monoclonal antibodies which inhibited human sperm binding to the ZP [46, 47]. Inhibition was based on the cross-reactivity of ZP between pigs and humans. MAb-5H4 (characteristic properties shown in Table 4) was reacted with an 18 mer synthetic peptide of pig ZPA (50–67) which had been selected by epitope mapping and flexibility analyses [48, 49].

To determine the minimum binding motif for MAb-5H4, a number of peptides were synthesized onto polypropylene pins by deleting one amino acid from either end of the 18 mer sequence. Modified ELISA revealed that the sequence 54–61 is essential for antibody binding (Fig. 1). The 8 mer sequence is essential but not sufficient for B-cell epitope since the synthetic 8 mer peptide was not recognized by MAb-5H4. Additional amino acids located near the 8 mer sequence seemed to be necessary for recognition by MAb-5H4. The 54–61 amino acid sequence was determined to be LDPENTL; N (aspargine) at position 58 is replaced with K (lysine) in rabbits and humans. That may be the reason why a higher MAb-5H4 concentration (10 times) is necessary for immunofluorescent staining of rabbit and human ZP as compared to pig ZP (unpublished data).

3) Inhibition of pig IVF by anti-pig type peptide

A pig 18 mer synthetic peptide of CTYVLDPENTLKAPYEA (50–67) was synthesized and conjugated to diphtheria toxoid (DT) with malemidecaproic acid for immunization in rabbits. The raised antiserum reacted not only with pig ZP but also rabbit and human ZP, suggesting that the synthetic peptide retained a native antigenic structure. When 10% antiserum was added to a medium of pig IVF, a significant inhibition of fertilization was observed compared to the control anti-DT antiserum (Table 5). In contrast to the pig system, no effects on rabbit or human IVF were observed.

As mentioned above, the replacement of one amino acid at position 58 of the synthetic 18mer peptide seemed to decrease its affinity with MAb-5H4. This was confirmed by the fact that the 18 mer synthetic peptide conjugated with DT was effective for blocking of fertilization only in pigs but not in humans. These results indicate that homologous antigens to target animals are necessary for the development of effective contraceptive vaccines.

As a next step, rabbit-type 18 mer peptide was synthesized and used for immunization in rabbits to produce high affinity antibodies to rabbit and human ZP.

4) Inhibition of human IVF by anti-rabbit type peptide

An 18 mer rabbit peptide homologue to pig 50–67

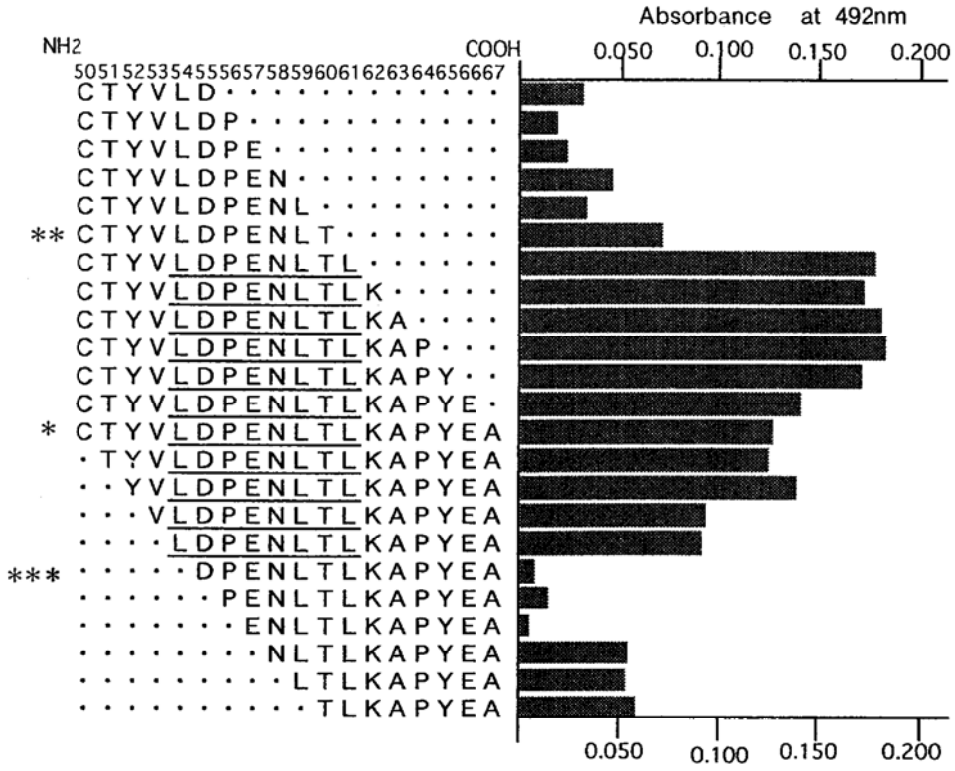

Fig. 1. Determination of the minimum binding motif. Peptides were synthesized on polypropylene pins by removing amino acids one by one from the 18-amino acid peptide (*CTYVLDPENLTLKAPYEA). Specific binding to MAb-5H4 decreased markedly in the peptide (**CTYVLDPENLT) from which amino acids 61-67 had been deleted at the COOH end and in the peptide (***DPENLTLKAPYEA) from which amino acids 50-54 had been deleted at the NH_2 end. This indicates that the 8-amino acid motif of 54-61 LDPENLTL is essential for the binding with MAb-5H4.

Table 5. Effect of addition of antiserum to synthetic peptide (pig type) on pig *in vitro* fertilization

Antisera	No. of eggs used	No. of eggs fertilized*	%	
DT**	16	7	43.8	***
peptide-DT	24	1	4.2	

*Enlarged sperm heads were observed in cytoplasm in fertilized eggs. **DT: Diphtheria toxoid. ***$p<0.01$ (χ^2 test).

Table 6. Effect of addition of antiserum to synthetic peptide (rabbit type) on human *in vitro* fertilization

Antisera	No. of eggs used	No. of sperm bound to ZP/egg (Mean ± S.D.)		No. of sperm penetrating into ZP/egg (Mean ± S.D.)
DT*	12	57.9 ± 24.3	**	2.8 ± 3.8
peptide-DT	14	3.6 ± 3.8		0 ± 0

*DT: Diphtheria toxoid. **$p<0.01$ (t test).

was synthesized in which the B cell epitope sequence was different only at position 58 (i.e. asparagine in pigs, lysine in rabbits). Anti rabbit 18 mer peptide antiserum was produced with the same protocol used to produce the pig 18 mer peptide. The antiserum reacted not only with rabbit ZP but also with human and pig ZP. As expected, the antiserum inhibited human sperm binding to human ZP. The antiserum had high affinity for human ZP probably because the B-cell epitope of human peptide 50–67 is identical to rabbit, but the antiserum did not inhibit rabbit IVF. We do not know the reason for this, but it may be due to the uniqueness of the rabbit fertilization mechanism. In rabbit fertilization, the ZP seems to play a less important role as a barrier to sperm penetration. It is well known that in rabbits large number of sperm penetrate the ZP and move into the perivitelline space. This indicates that rabbit ZP does not function as a barrier to polyspermy [50].

5) Production of autoantibody to ZP

One of the most important points in developing contraceptive vaccines is that the synthetic zona peptide can induce autoantibodies to the ZP in immunized animals. In our study an 18mer synthetic peptide 50–67 from rabbit ZPA evoked autoantibodies reactive to rabbit ZP [51]. Immunoglobulin was detected on the ZP in ovarian sections from the immunized rabbits without any morpholigical changes including impairment of growing follicles and depletion of primordial follicles noted in the histological study. This suggested that rabbit autoantibodies produced by active immunization with synthetic rabbit ZP peptides could bind the native epitope of the ZP *in vivo*. These results also indicate that B cell clones for self ZP antigens are not completely eliminated. The fact that autoantibodies to a synthetic ZP peptide could be produced by conjugation to a strong foreign T-cell epitope such as DT indicates the possibility of developing contraceptive vaccines by using protein engineering technology. In addition, if a single B cell epitope does not induce a sufficient blocking effect, a multivalent vaccine having several specific and effective epitopes in a single formulation may provide an ideal vaccine regimen [52].

Conclusion

Recombinant ZP proteins with or without carbohydrate chains elicited antibodies reactive to native ZP concomitantly with induced ovarian dysfunction. To avoid this undesirable effect, a peptide sequence of ZP which does not contain cytotoxic T cell epitopes must be defined. We found that the NH_2-terminal region of pig ZPA could produce fertilization blocking antibodies [39]. Antiserum to a recombinant pig ZPA corresponding to the 198 amino acid NH_2-terminal sequence strongly blocked pig IVF but not human IVF. In contrast, antiserum to a recombinant protein of 206 amino acids, which corresponds to the NH_2-terminal region of human ZPA, was able to inhibit human sperm binding to the ZP. These results suggested that a homologous system must be used for induction of fertilization blocking antibodies. This means that the amino acid sequence for immunization must be the same as the target animal ZP protein sequence. Fortunately the sequence homology of ZP proteins is conserved to a high degree in primates, so the non-human primate would be the best model for predicting how humans would respond to the ZP vaccine.

B cell epitope is usually well-defined in synthetic peptide vaccines compared to the recombinant vaccines, although a foreign T cell epitope has to be conjugated to the defined B cell epitope. Monoclonal antibodies capable of interfering with the sperm-zona interactions will help in defining promising B cell epitopes. Our previous data suggested that the synthetic peptide of pig ZPA (50–67) could be used as a contraceptive vaccine since the sequence was recognized by a fertilization blocking monoclonal antibody (MAb-5H4). Antiserum to the synthetic pig peptide blocked the fertilization in pigs, but not in humans, but antiserum to the synthetic rabbit peptide, in which the minimum binding motif for MAb-5H4 is identical to that of humans, could interrupt human sperm-zona interaction. These results indicated that species cross-reactive antigens may not be sufficient to induce antibodies with high affinity. It was suggested that an antigen with a sequence homologous to the target animal ZP proteins should be used to induce highly effective antibodies for blocking fertilization both in recombinant and peptide vaccines. Although experiments on non-human primates are necessary to better understand vaccine efficacy *in vivo* as well as any effects on ovarian functions before use in humans, the present evidence encourages us to continue our research on developing a contraceptive vaccine by using ZP antigens.

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