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DEVELOPMENT OF AN IMMUNOCONTRACEPTIVE VACCINE FOR BIOCONTROL OF RATS

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DEVELOPMENT OF AN IMMUNOCONTRACEPTIVE VACCINE FOR BIOCONTROL OF RATS

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Chairman: **Professor Mohd. Azmi Mohd. Lila, Ph.D.**

Faculty: **Veterinary Medicine**

Zona pellucida (ZP) is an extracellular matrix that surrounds the mammalian oocyte. The rat ZP is composed of three major glycoproteins: ZP1, ZP2 and ZP3. As a primary sperm receptor, ZP3 has been utilised as an immunogen to prevent fertilisation of the ovum. Unfortunately, the availability of ZP3 protein is always limited and purified protein is not available. DNA vaccination is therefore an excellent alternative.

ZP3 cDNA was amplified from *R. rattus diardii* ovary as a first step towards the development of ZP3-based DNA vaccine. The ZP3 gene has an open reading frame of 1272 nucleotides encoding a polypeptide of 424 amino acid residues which shares 87% identity with mouse homologue. The anti-fertility polynucleotide vaccine was generated by placing ZP3 gene into a mammalian plasmid expression vector. Plasmid containing the entire ZP3 gene sequence was designated pcDNA/1300. Meanwhile, constructs pcDNA/720 and pcDNA/580 comprised partial ZP3 gene encoding the N-terminal and C-terminal half of the protein respectively. *In vitro*

transfection of mammalian cells with these plasmids DNA led to their cytosolic expression. Sperms were attracted and bound to cells harbouring pcDNA/1300 and pcDNA/580 because these two constructs encoded the sperm-combining sites of ZP3.

Administration of these DNA vaccines resulted *in vivo* expression of ZP3 protein, which in turn stimulates specific cellular and humoral immune responses directed against self-ZP3 protein bearing cells or oocytes. Lacking or destruction of such cells resulted in an effective contraception in female animals. The ovarian dysfunction was characterised by excessive depletion of follicles and an increase in the number of oocyte-free cell clusters. The integrity of most follicles was challenged, as it was significantly infiltrated by immune cells. These ZP3 specific immune cells were shown to be a mixture of CD4+ and CD8+ T-lymphocytes subsets. Alterations in ovarian function were also evidenced when vaccinated animals were no longer sensitive to an intensive exogenous hormonal (hCG) treatment. Among the three constructs, pcDNA/1300 is the most effective contraceptive vaccine followed by pcDNA/580. The reduction in average litter size achieved by pcDNA/1300 was >90%. It is an excellent irreversible contraceptive vaccine as none of the vaccinated rats showed signs of recovery after three injections. In contrast, rats vaccinated with pcDNA/580 regained fertility over an extended period. On the other hand, vaccination with pcDNA/720 construct has no significant impact on rat fertility. Hence, special attention was given to pcDNA/1300.

In DNA vaccination, the role of cell-mediated immune response was pre-eminent as the titre of ZP3-antibody produced was significantly low. Relatively, vaccination

with recombinant ZP3 protein expressed by yeast cells, *P. pastoris* stimulated strong antibody response, but no correlation between antibody titres and infertility was observed. Similarly, when the cytokines IL-4 gene was co-immunised along with pcDNA/1300, a dramatic increase in ZP3 antibody level did not enhance the efficacy of pcDNA/1300. Indeed by driving the immune response of pcDNA/1300 towards Th2 direction, this weakened its effectiveness in preventing fertilisation.

Construct pHumoral-ZP3 was assembled as an attempt to improve the potency of pcDNA/1300. Modification of pcDNA/1300 with viral NP conjugation produced a significant enhancement on the levels of ZP3 antibody. The magnitude of the antibody response was comparable to that generated through the use of cytokine genetic adjuvant, IL-4. However, despite high ZP3 antibody titres, rats vaccinated with pHumoral-ZP3 produced normal litter size.

This study demonstrated the application of a ZP3-based DNA vaccine in fertility control. The results obtained are extremely encouraging for the development of a vaccine for lasting rat population control. Meanwhile, the current DNA construct serves as excellent model for the generation of similar vaccines to prevent individual animals from conceiving. As a result, the expensive and complicated invasive procedures like surgery and castration can be avoided.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBINAAN VAKSIN PENCEGAH PENGHAMILAN UNTUK BIO KAWALAN TIKUS

Oleh

LAI KIT YEE

Mei 2004

Pengerusi: **Profesor Mohd. Azmi Mohd. Lila, Ph.D.**

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Zona palusida (ZP) adalah matriks ektraselular yang mengelilingi oosit mamalia. ZP tikus terdiri daripada tiga glikoprotein utama: ZP1, ZP2 dan ZP3. Selaku reseptor sperma primer, ZP3 telah digunakan sebagai imunogen untuk mencegah persenyawaan ovum. Namun, protein ZP3 yang tersedia ada adalah terhad dan protein yang ditulinkan tidak tersedia ada. Oleh itu, penvaksinan DNA adalah alternatif terbaik.

ZP3 cDNA telah diamplify dari ovari *R. rattus diardii* dalam langkah permulaan membangunkan vaksin DNA berasaskan ZP3. Gen ZP3 mempunyai kerangka bacaan terbuka (ORF) 1272 nucleotida yang mengkodkan polipeptida sepanjang 424 asid amino yang mana berkongsi 87% persamaan dengan homolog mencit. Vaksin polinukleotida anti-subur dihasilkan dengan menempatkan gen ZP3 ke dalam vector pengekspresan plasmid mamalia. Plasmid yang mengandungi kesuluruhan jujukan gen ZP3 dinamakan pcDNA/1300. Sementara itu, binaan pcDNA/720 dan pcDNA/580 masing-masing mengandungi separuh dari bahagian gen ZP3 yang

mengkodkan terminal-N dan terminal-C. Transfeksi in-vitro sel mamalia dengan plasmid DNA ini menyebabkan ekspresi sitosol. Sperma-sperma tertarik dan terikat kepada sel yang mengandungi pcDNA/1300 dan pcDNA/580 kerana kedua-dua binaan ini mengkodkan tapak pelekatan sperma ZP3.

Pengambilan vaksin-vaksin DNA ini mengakibatkan ekspresi protein ZP3 in-vivo, seterusnya merangsangkan tindakbalas imun sel-sel khusus dan humoral menentang sel-sel yang mengandungi protein ZP3 ataupun oosit-oosit. Kekurangan atau kemusnahan sel-sel seperti ini menyebabkan penghamilan pada haiwan betina dicegah dengan berkesan. Kegagalan fungsi ovari dicirikan dengan penghabisan melampau folikel-folikel dan meningkatnya bilangan kelompok-kelompok sel oosit bebas. Integriti kebanyakan folikel ternyata dicabar oleh sel-sel imun yang menyusup masuk. Sel-sel imun khas ZP3 ini dikenalpasti sebagai campuran subset T-limfosit CD4+ dan CD8+. Perubahan dalam fungsi ovari turut dibuktikan apabila haiwan yang divaksinkan tidak lagi peka kepada ransangan hebat hormon (hCG) luar. Antara ketiga-tiga binaan, pcDNA/1300 adalah vaksin pencegah penghamilan yang paling berkesan diikuti dengan pcDNA/580. Pengurangan saiz purata anak yang dicapai oleh pcDNA/1300 adalah >90%. Ia adalah vaksin pencegah penghamilan tidak keterbalik yang bagus kerana tiada tikus-tikus yang divaksin menujukkan tanda pulih selepas tiga kali suntikan. Sebaliknya, tikus-tikus yang divaksinkan dengan pcDNA/580 kembali subur apabila jangkamasa dilanjutkan. Dalam hal yang lain pula, penvaksinan dengan binaan pcDNA/720 tidak mempunyai sebarang kesan yang nyata terhadap kesuburan tikus. Maka, perhatian istimewa diberikan kepada pcDNA/1300.

Dalam penvaksinan DNA, tindakbalas imun dengan perantaraan sel memainkan peranan penting sebab titer antibodi ZP3 yang dihasilkan terlalu rendah. Secara relatifnya, penvaksinan dengan protein ZP3 rekombinan yang diekspreskan oleh sel-sel yis, *P. pastoris* meransang tindakbalas antibodi yang kuat. Akan tetapi, tidak pula dilihat perkaitan antara titer antibodi dan ketidaksuburan. Keadaan yang sama berlakuapabila gen sitokin IL-4 diko-imunkan bersama dengan pcDNA/1300, kenaikan paras antibodi ZP3 tidak juga meningkatkan keefisyenan pcDNA/1300. Sebetulnya, tindakbalas imun pcDNA/1300 dituju ke hala Th2 telah melemahkan keperkesanannya dalam menghalang persenyawaan.

Binaan pHumoral-ZP3 dianggap dapat memperbaiki potensi pcDNA/1300. Pengubahan pcDNA/1300 dengan konjugasi virus NP ternyata sekali telah menaikkan paras antibodi ZP3. Kadar tindakbalas antibodi adalah setanding dengan penggunaan genetik adjuvant sitokin, IL-4. Tetapi, walaupun titer antibodi adalah tinggi, tikus-tikus yang diberi vaksin melahirkan saiz anak yang normal.

Kajian ini menujukkan penggunaan vaksin DNA berdasarkan ZP3 dalam kawalan kesuburan. Keputusan yang diperolehi sangat menggalakkan dalam membangunkan vaksin untuk kawalan populasi tikus selamanya. Dalam pada itu, binaan vaksin DNA ini merupakan model yang sangat baik untuk menghasilkan vaksin yang serupa bagi mencegah individu binatang daripada mengandung. Dengan demikian, cara-cara pelanggaran yang mahal dan sulit seperti pembedahan pengembirian dapat dielakan.

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I certify that an Examination Committee met on 17 May 2004 to conduct the final examination of Lai Kit Yee on her Doctor of Philosophy thesis entitled "Development of An Immunocontraceptive Vaccine for Biocontrol of Rats" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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List of the standard one-letter amino acid codes and their three-letter equivalents.

Amino acid	One-letter notation	Three-letters notation
Alanine	A	Ala
Arginine	R	Arg
Asparagines	N	Asn
Aspartate	D	Asp
Cysteine	C	Cys
Glutamate	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cells
AOXI	Alcohol Oxidase I
BMGY	Buffered Glycerol-complex Medium
BMMY	Buffered Methanol-complex Medium
cDNA	Complementary Deoxyribonucleic Acid
CFA	Complete Freund's Adjuvant
CpG	Cytosine-Phosphate-Guanosine
CS-1	Cleavage Signal Protein
CTL	Cytotoxic T-lymphocytes
DAB	3'-3' Diaminobenzidine
D-MEM	D-Minimum Essential Media
EDTA	Ethylene Diamine Tetra-Acetate
ELISA	Enzyme Linked Immunosorbent Assay
FA-1	Fertilisation Antigen-1
FSH	Follicle-Stimulating Hormone
GDF-9	Growth Differentiation Factor 9
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GnRH	Gonadotrophin-Releasing Hormone
hCG	Human Chorionic Gonadotropin
HCMV	Human Cytomegalovirus
HRP	Horse Raddish Peroxidase
IFA	Incomplete Freud's Adjuvant
IFN-γ	Inteferon-γ
KLH	Keyhole Limpet Hemacyanin

LDH-C_4	Lactate Dehydrogenase C_4
LH	Luteinising Hormone
MHC	Major Histocompatibility Complex
NGS	Normal Goat Serum
NK	Natural Killer
NP	Nucleoprotein
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PH-20	Sperm Surface Protein (with hyaluronidase activity)
PVDF	Polyvinylidene Difluoride
RNA	Ribonucleic Acid
RT-PCR	Reversed Transcript Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis
SP-10	Sperm Protein-10
Tc1	Type 1 Cytotoxic Cells
Tc2	Type 2 Cytotoxic Cells
TE	Tris-EDTA
TEM	Transmission Electron Microscopy
TGFβ	Transforming Growth Factor β
YPD	Yeast Extract Peptone Dextrose Medium
ZP	Zona Pellucida

CHAPTER I

INTRODUCTION

For many years, rats have been a persistent problem to the country. Current control methods such as trapping, shooting and poisoning are not long-term solutions because of the intrinsic capacity of rats to rapidly replace those killed. Curbing the reproductive potential of the pest therefore is a more effective way to control it. Immunocontraception is a new concept of controlling fertility by using a vaccine. It aims to trick the host's immune system into generating an immune reaction against cells or molecules that are essential for reproduction, such as hormones (egs. FSH and LH) that trigger the maturation and release of egg cells in the female or a continuous production of sperms in the male, or against the gametes themselves or against pregnancy-related hormones (eg. progesterone). If successful, this approach will effectively reduce the rat population density. It is also less costly, more humane and environmental friendly as compared to existing methods, mentioned earlier.

There are three major steps toward the development of a contraceptive vaccine: selecting a potential agent to deliver the vaccine, identifying the antigen to use in the vaccine, and ensuring that the vaccine is species-specific. In an earlier study (Lai *et al*. 1998), rat cytomegalovirus (RCMV) was identified as a suitable vehicle to carry the vaccine. A narrow host range, the capability of establishing latent and persistent infections, and having a large DNA genome are some of the main features that make this virus a promising vector for fertility-associated genes. Following the above strategies, the next mission is to isolate and characterise the relevant antigen,

reassemble it into functional vaccine and conduct studies to evaluate its efficacy before incorporating it into the delivery system.

Basically, target antigens for immunocontraception can be grouped into two categories: reproductive hormones and gamete proteins. In the present study, the latter are preferred because they are tissue-specific and non-circulatory; complications arising from an immune complex formation should not happen. Evidence to support this hypothesis is found in nature. Several clinical studies have shown the association of antibodies against sperm antigens with an otherwise unexplained infertility (Scarselli *et al*. 1973; Mettler *et al*. 1974; Ingerslev, 1981; Witkin and David, 1986). Autoantibodies against the egg protein, zona pellucida (ZP) have also been documented (Nishimoto *et al*. 1980; Buckshee and Mhaskar, 1985; Kamada *et al*. 1992) in infertile patients that otherwise appear healthy, indicating that an immunological block to fertility is prevalent in the human situation, without any side effects. The focus is finally given to egg proteins because in most species, the ova surfaces consist of only three proteins and their individual functions are well understood compared to sperm antigens.

Zona pellucida is an acellular translucent matrix that surrounds the mammalian oocytes. It serves as the docking site for initial recognition and binding of the sperm to the oocyte in a species-specific manner, induces the acrosome reaction in the zona-bound sperm, blocks polyspermy, and protects the pre-implanted blastocyst. The critical involvement of ZP in the fertilisation process, together with its tissue-specific nature, has made it one of the promising target antigens for the development of an immunocontraceptive vaccine. Molecular composition analysis has revealed

that the ZP extracellular matrix is composed of three major glycoproteins with high homology among species (Harris *et al*. 1994; Zhu and Naz, 1999). These glycoproteins have been named as ZP1, ZP2 and ZP3 based on their mobility in SDS-PAGE. In rodents, ZP3 acts as a primary sperm receptor with a specific class of O-linked oligosaccharides involved in sperm binding (Wassarman, 1990).

The use of ZP as a possible means of birth control has long been investigated. Porcine ZP has been extensively utilised in these studies due to its high availability at the abattoirs. Immunisation with porcine ZP has resulted in significant zonae antibody titres and inhibition of fertility in a number of species including rabbits (Skinner *et al*. 1984), hamsters (Hasegawa *et al*. 1992), dogs (Mahi-Brown *et al*. 1988), primates (Paterson *et al*. 1992; Bagavant *et al*. 1994), horses (Kirkpatrick *et al*. 1990, 1996) and deer (Miller *et al*. 1999, 2000). Unfortunately, rats and mice immunised with the identical immunogen remained fertile (Wood *et al*. 1981; Sacco *et al*. 1981). Hence, 'self' ZP is required to suppress fertility in these animals. Similar to porcine ZP, the murine ZP (mZP) has been explored in some details. The mZP3 antigens either in the form of peptides or recombinant proteins have been demonstrated to induce antibody-mediated contraception (Sacco, 1979; East *et al*. 1985; Miller *et al*. 1989; Zhang *et al*. 1997) as well as autoimmune oophoritis in mice (Rhim *et al*. 1992; Lou *et al*. 1996). In contrast, little information is available on rat ZP, particularly, its use in immunocontraception. However, based on the established knowledge of mZP, the present study has been designed to develop a special contraceptive vaccine to control the rat population. Genetic vaccination can lead to active protein production *in vivo* and induces a broad spectrum of immune responses (Ulmer *et al*. 1993; Kowalczyk and Ertl, 1999). As such, hypothetically, a