COMPARISON OF PIG AND RABBIT ZONAE PELLUCIDAE VACCINATION IN THE FEMALE BABOON (*Papio anubis*): ANALYSIS OF IMMUNOGENICITY AND OVARIAN PATHOLOGY.

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MARY J. A. OGUNA, BVM,

DEPARTMENT OF ANIMAL PHYSIOLOGY,

UNIVERSITY OF NAIROBI, KENYA.

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE MASTER OF SCIENCE DEGREE IN REPRODUCTIVE BIOLOGY OF THE UNIVERSITY OF NAIROBI, KENYA.

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ABSTRACT

Immunocontraception using zona pellucida (ZP) antigens has been considered to have great potential. However, the variations in the immune response to ZP proteins in different species is great. Since little is known about the immune responses to ZP of different species in non-human primates, an experimental study was conducted to compare the immunogenicity of native rabbit ZP and native pig ZP in the female baboon (*Papio anubis*) and to evaluate the effect of immunization on ovarian function.

Eleven cycling female baboons were randomly divided into three groups comprising: (1) three controls immunized with TitreMax® adjuvant alone; (2) four females immunized with 450 µg each of pig ZP in TitreMax® adjuvant and; (3) four females immunized with 450 µg each of rabbit ZP in TitreMax® adjuvant. Each animal was immunized three times (150 µg immunogen at each immunization) at 21-day intervals. Blood, collected at the time of each immunization and monthly thereafter, was examined for the presence of antibodies against rabbit, pig and baboon ZP. Unilateral ovariectomies were initiated at 50 days after the primary immunization, and the ovaries subjected to histological analysis. Ovarian cyclicity was monitored by daily scoring of sex skin swelling.

ELISA results showed that antibodies were produced against the respective immunogen (native rabbit or pig ZP) with titres being higher for the rabbit ZP group. The ELISA results also showed that sera from the rabbit ZP-immunized group recognized native pig ZP and sera from the pig ZP-immunized group recognized

native rabbit ZP. In addition to verifying the ELISA results, immunoblot analysis also revealed that the antisera recognized bacterially produced recombinant (BPR) rabbit ZP antigen. Interestingly, sera from all pig ZP-immunized animals only recognized BPR protein encoding the 75a portion of the 75-kDa rabbit ZP protein, while sera from each animal immunized with rabbit ZP recognized all the recombinant rabbit ZP proteins tested (55-kDa and 75a and 75b portions of the 75-kDa protein). Immunohistochemistry on sections of normal baboon ovary probed with immune baboon sera against rabbit or pig ZP demonstrated the induction of auto-antibodies against baboon ZP. Histological analysis of ovaries removed from immunized animals did not indicate any overt pathology, and all animals continued to cycle normally, as evidenced by scoring of sex skin swelling, for the 12 months of the study.

Collectively, these results show that heteroimmunization of female baboons with either native rabbit ZP or native pig ZP in TitreMax® adjuvant results in the production of antibody populations that not only recognize their respective immunogen and native baboon ZP but also BPR protein encoding portions of the rabbit ZP antigen. In addition, these antibody populations recognize the cross-reactive native ZP (that is pig ZP for the rabbit ZP-immunized group and vice versa). None of these antibody populations result in any overt ovarian pathology.

It can therefore be concluded that there is a difference in terms of immunogenicity and none in terms of ovarian pathology between immunization with native pig ZP and native rabbit ZP using TitreMax® adjuvant in female baboons. However, it would also be important to evaluate the effects of these antibody populations on sperm-egg binding and *in vivo* fertility.

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

....

MARY J. A. OGUNA, BVM,

Department of Animal Physiology,

University of Nairobi, Kenya.

This thesis has been submitted for examination with our approval as University supervisors.

E. D. SCHWOEBEL, BA, PhD, **DATE**

Programme Leader, Molecular Biology,

Institute of Primate Research, Kenya.

E. O. WANGO, BVM, MSc, PhD, **DATE**

Senior Lecturer, Department of Animal Physiology;

Coordinator, MSc Reproductive Biology Programme

and Reproductive Biology Unit,

University of Nairobi, Kenya.

G. Otiang'a-Owiti .... 11 March 1997 ....

G. E. OTIANG'A-OWITI, BVM, MSc, PhD, **DATE**

Senior Lecturer and Chairman,

Department of Veterinary Anatomy,

University of Nairobi, Kenya.

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DEDICATION

TO:

MY FAMILY AND FIANCÉ

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ABBREVIATIONS AND SYMBOLS

APS	Ammonium persulphate
BPR	Bacterially produced recombinant
BPRP	Bacterially produced recombinant protein
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
CH	Corpus hemmorhagicum
CL	Corpus luteum
cm	Centimetre
Co.	Company
Corp.	Corporation
DAB	3', 3-Diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Fc	Fraction crystallizable
g	Gram
H_2O	Water
H_2O_2	Hydrogen peroxide
IFA	Incomplete Freund's adjuvant
IgG	Immunoglobulin G
IPR	Institute of Primate Research

IUD	Intrauterine device
IVF	*In vitro* fertilization
KCC	Kenya Cooperative Creameries
KCl	Potassium chloride
kDa	Kilodalton
kg	Kilogram
KH_2PO_4	Monobasic potassium phosphate
KLH	Keyhole Limpet Hemocyanin
M	Molar
m^2	Square metre
MDP	Muramyl dipeptide
mg	Milligram
ml	Millilitre
mM	Millimolar
mm^3	Cubic millimetre
MO	Missouri
NaCl	Sodium chloride
$Na_2CO_3.H_2O$	Sodium carbonate monohydrate
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Monobasic sodium phosphate
$Na_2HPO_4.7H_2O$	Dibasic sodium phosphate heptahydrate
$NiCl_2$	Nickel chloride
NJ	New Jersey

OPD	o-Phenylenediamine dihydrochloride
PA	Philadelphia
PBS	Phosphate buffered saline
PZP	Pig zona pellucida
rec	Recombinant
RZP	Rabbit zona pellucida
SDS	Sodium dodecyl sulfate
SPLPS	Sodium phthalylated lipopolysaccharide
TEMED	N, N, N', N'-Tetramethlylenediamine
TTM	TitreMax® adjuvant
USA	United States of America
WHO	World Health Organization
ZP	Zona pellucida
1D-PAGE	One dimensional-polyacrylamide gel electrophoresis
1D, 2D SDS-PAGE	One-, two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
1X	One times
10X	Ten times
2-ME	2-Mercaptoethanol
°C	Degrees centigrade
ηg	Nanogram
ηm	Nanometre
µg	Microgram

µl	Microlitre
µm	Micrometre
®	Registered trade mark
/	Per
%	Per cent

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Despite recent epidemiological evidence that adjusted fertility rates are falling in some countries, and that the world population is not increasing as rapidly as feared, the rate of increase is still high enough to bring the global figure to 8 billion by the year 2052 (Spira, 1994), even according to the most conservative of projections. The overall world population growth rate approximates 1.7% per annum-a yearly natural increase of 72 million and a doubling time of 42 years (Lincoln, 1992). As the human population grows, more and more land is required for homes and fields. This brings humans into direct conflict with wild animals. The populations of the latter have also been showing a similar trend of increase. Domestic animals have not been spared either; tens of thousands of unwanted dogs and cats are euthanized each year as a population control measure (Dunbar and Schwoebel, 1988). Proper control of both human and animal populations is therefore imperative.

Many population control measures do exist. However, the acceptance of a need to develop a new and unique contraceptive technology has stemmed from a recognition of the inadequacies of present technology, and the gaps in the choice of available contraceptive methods. There is no method of fertility regulation that does not have substantial disadvantages (Lincoln, 1992)-hence the need to develop newer methods. Immunoregulation of fertility represents such a potential technology. The principle of immunological contraception is inherently attractive, involving as it does, the concept of manipulation of the body's natural defenses to block or interrupt the

reproductive process. The basis of this principle is that the reproductive process has components that are both unique and immunogenic, thereby raising the possibility that they may be susceptible to a relatively precise immunological attack. One such component is the mammalian zona pellucida (ZP).

The basic attractiveness of an approach involving the ZP relates to its accessibility, as a limited target, to the effects of antibodies during the several days it is present free in the reproductive tract (Yanagimachi, 1994). However, its presence around the secondary oocytes in the tissues of the ovary detracts from the precision of an immune attack. This latter fact raises certain disadvantages, chief of which is impairment of ovarian function. It has therefore become critical to begin to evaluate those forms of zonae pellucidae that can be used to generate antibodies that will specifically interfere with fertilization without altering ovarian function.

1.2 Literature review

1.2.1 Population statistics

" If all the people of the world are to enjoy the highest possible level of health and basic human rights, it is imperative that research on contraceptive development continues unhindered. Moreover, without such research it would be difficult for the world to bring its population and natural resources into a sustainable balance." This was part of the declaration (cited in Khanna *et al.*, 1994) adopted by the participants in the International Symposium on Contraceptive Research and Development for the year 2000 and Beyond, held in Mexico city in 1993. Indeed, overpopulation today is widely acknowledged to be the world's single most pressing health problem despite recent epidemiological evidence that the overall world population growth rate is

decreasing. The current growth rate, however, is still high enough to bring the global figure to six billion by the year 2000 (Dunbar and Schwoebel, 1988; Dirnhofer *et al.*, 1994) and ten billion by the year 2100 (Spira, 1994), even according to the most conservative of projections; 95% of this growth will be located in the economically and environmentally fragile regions of the Southern Hemisphere (Lincoln, 1992). This increase undoubtedly, will strain the already stressed markets in food, housing, labour, sanitation and basic resources. The overall world population growth rate approximates 1.7% per annum-a yearly natural increase of 72 million and a doubling time of 42 years (Lincoln, 1992).

This trend is indeed disquieting considering that half of all pregnancies are unplanned and a quarter certainly unwanted (Senanayake, 1994). The latter is a major public health problem with potentially serious consequences for the health of the girl or woman. Not only is it a denial of a woman's fundamental right to control her fertility, but it also exposes her to the hazards of pregnancy and childbirth. In developing countries 1 in 50 women dies from complications of pregnancy and childbirth, compared to only 1 in 2700 in developed countries (Khanna *et al.*, 1994). In addition, many unwanted pregnancies result in abortion. Daily, around 140,000 (or annually, 50 million) abortions are performed around the world (Lincoln, 1992). In developing countries many pregnancies are terminated in clandestine or otherwise unsafe conditions. This exposes the woman to a high risk of mortality and morbidity. The estimated annual number of unsafe abortions in the world is 21 million and at least 180 women die every day from unsafe abortions (Khanna *et al.*, 1994). At the home level, the death of a woman is devastating to her family and more so to her

children under five years of age whose chances of death increase by 50% (Khanna *et al.*, 1994). Problems arising from increased fertility are not only experienced at the family level but also at the global level.

As the human population grows, more and more land is required for homes and fields. This brings humans into direct conflict with wild animals whose populations have also been showing a similar trend of increase. In non-industrialized regions such as Sub-Saharan Africa, persistent high fertility and rapid population growth are associated with deterioration of both the biotic and abiotic environments. The epidemiological consequences of such environmental deterioration are a rapidly growing facet of medical treatment and public health problems. In a bid to alleviate the situation, some wildlife populations, predisposed toward overpopulation, are periodically culled (Kirkpatrick and Turner, 1991; Turner *et al.*, 1992). Domestic animals are not spared either; for pet overpopulation is one of the most serious problems in the field of animal protection. To this end, tens of thousands of unwanted dogs and cats are euthanized each year (Dunbar and Schwoebel, 1988). Control of both human and animal populations is therefore imperative.

1.2.2 Prevalence of contraception

Despite the wide range of currently available contraceptive methods, the prevalence of their usage (defined as the percentage of married women of reproductive age or their husbands, using any form of contraception) is still low, and alarmingly so in developing countries. In 1990, up to 57% of all married women of reproductive age or their husbands were using a method of contraception (Khanna *et al.*, 1994). This figure is not much different from the 1987 figure of 53% (Spira,

1994) and shows a worrying trend in that there is no significant increase. Though the overall world contraceptive prevalence of 57% was encouraging, it however masked the situation in the least developed countries, representing some 540 million people, where the total fertility rate (defined as the average number of children per woman) was still 6.1 births per woman in 1992, corresponding to an estimated prevalence of contraceptive use of only 14%. It is estimated that there are some 120 million women in developing countries who are not practicing family planning even though they say that they do not want to become pregnant (Khanna *et al.*, 1994). On the global level, current estimates are that 200-500 million couples wish to plan their families but lack adequate contraceptive choices or access to quality services (Senanayake, 1994). This number will probably increase, particularly in developing countries where the number of married women of reproductive age is projected to grow by approximately 28%, from 747 to 959 million between the year 1990 and 2000 (Senanayake, 1994). Indeed, the current methods of contraception were largely developed in the 1950s and 1960s. Collectively these methods can limit population growth although their acceptability at a personal level remains marginal. Table 1 below summarizes the contraceptive prevalence in 1987.

Table 1 Average *prevalence of specific contraceptive methods, by region, 1987 (adapted from Spira, 1994)

Region	All Methods	Sterilization : Female	Sterilization : Male	Pill	Injectable	IUD	Condom
World	53	16	4	7	1	11	5
More developed regions	71	8	4	14	-	6	13
Less developed regions	48	18	5	5	1	12	3
China	72	28	8	3	0.2	30	2
Other countries	38	14	3	6	1	4	3
Africa	17	1	-	7	1	3	1
Northern Africa	31	2	-	16	0.3	8	1
Sub-Saharan Africa	13	1	-	4	2	1	0.5
Asia and Oceania	53	21	6	4	1	14	3
East Asia	72	28	8	3	0.2	29	2
Other countries	40	16	5	4	1	4	4
Latin America	57	20	1	16	1	6	2

Key

* : Note that individuals may use more than one type of contraceptive concurrently and so the total prevalence of any one method may add to more than 100%

IUD : Intrauterine device

This low prevalence of contraceptive use points to the inadequacies of present technology and the gaps in choice of available contraceptive methods. An increasing number of users in both developed and developing countries are dissatisfied with the currently available options for a number of reasons that include: side-effects, unreliability, long-term safety concerns, inconvenience, storage and disposal difficulties, irreversibility and high cost. What are missing from the contraceptive arsenal, therefore, are methods that do not produce the endocrine and metabolic disturbances associated with some steroidal contraceptives, are free of the inconvenience of daily pill taking, do not present logistical problems of storage and disposal that are associated with some barrier methods, do not require the insertion of a device, do not require surgical intervention, are relatively long-lasting but not permanent, and are inexpensive to manufacture and of low cost to the user. The need to develop a new and unique contraceptive technology (of which immunoregulation of fertility is one) that will answer most if not all of the above requirements can therefore not be denied.

1.2.3 Immunoregulation of fertility

Immunoregulation of fertility or immunocontraception is defined as the prevention of fertility by immunologic methods. The rationale for a fertility regulating vaccine has been established for many years by studies of the induction of immunological infertility in immunized experimental animals and by the recognition of the role of immunological factors in a proportion of infertile men and women (Lincoln, 1992). In addition, the reproductive process has components, such as the

mammalian ZP, that are both unique and immunogenic. This raises the possibility that they may be susceptible to a relatively precise immunological attack.

Due to these concepts, a great deal of emphasis has been placed on immunological methods for regulation of fertility (Griffin, 1991, 1996). There is no doubt that, viewed in the light of the escalating appraisals of the long-term safety and acceptability of currently available contraceptive methods, the principle of immunological contraception has certain well-defined potential advantages (Aitken *et al.*, 1993; Jones, 1994). These include: (1) the use of a 'non-pharmacologically active' agent; (2) ease and convenience of administration, making it suitable for distribution, if necessary, by paramedical personnel; (3) a long lasting (up to 12 months or more) but potentially reversible effect; (4) acceptability of the 'vaccine' principle- of particular importance in developing countries where the method can be incorporated into primary health care programmes; (5) reduced patient failure and (6) the possible large-scale synthesis and manufacture of vaccines at low cost. A variety of antigens have been considered for use as vaccines for immunological contraception in both humans and animals. These include hormone antigens (Griffin, 1983; Raj, 1983), sperm antigens (Alexander and Isahakia, 1983; Anderson, 1983) and ZP antigens (Aitken *et al.*, 1984, 1996; Skinner *et al.*, 1996).

1.2.4 Structure and function of the zona pellucida

The ZP is a unique extracellular sulfated glycoprotein matrix that is formed during the growth and maturation of mammalian oocytes (Dunbar and Wolgemuth, 1984; Maresh *et al.*, 1990). This relatively thick, transparent extracellular coat surrounds growing oocytes, ovulated eggs and preimplantation embryos. While

primordial oocytes do not have a ZP, the ZP glycoproteins represent a major secretory product of growing follicles. Depending on the species, the secretion of the ZP is either solely by the oocyte or in conjunction with the granulosa cells (Yanagimachi, 1994). In the mouse for example, expression of each ZP gene is restricted to the oocyte (Epifano *et al.*, 1995) while in the rabbit the follicular cells are also involved in the formation of the ZP (Wolgemuth *et al.*, 1984; Maresh *et al.*, 1990). The ZP matrix first appears as amorphous material deposited in the space between the oocyte and the surrounding granulosa cells. This material is subsequently assembled into long filaments forming a highly porous matrix that increases in thickness to 7 μm in fully grown mouse oocytes (Philips and Shalgi, 1980). In other species, the thickness ranges from 1-2 μm in the opossum, 13 μm in the human, 16 μm in the pig to 27 μm in the cow (Dunbar *et al.*, 1994; Yanagimachi, 1994).

Of late, the ZP has attracted considerable attention, since from its appearance during oogenesis to its removal by the embryo just prior to implantation, this extracellular coat plays a multifaceted role in mammalian development (Aitken *et al.*, 1984). Numerous investigations in various species have revealed that it is composed of three to four developmentally regulated glycoproteins (Saling, 1991; Bagavant *et al.*, 1993; Hinsch *et al.*, 1994) that serve essential functions during growth and development of the ovarian follicle (Dunbar and Schwoebel, 1988; Skinner *et al.*, 1990), species-specific attachment and binding of spermatozoa to oocytes (Shur and Hall, 1982; Brown and Jones, 1987; O'Rand, 1988; Yonezawa *et al.*, 1995), induction of the sperm acrosome reaction (Bleil and Wassarman, 1985; Saling, 1991), the block to polyspermy in some species (Storey and Kopf, 1991) and protection and

development of the preimplantation embryo (Dunbar and Wolgemuth, 1984; Wassarman, 1988; Suzuki *et al.*, 1995). For example, in the mouse and human, this extra-cellular matrix is composed of three major glycoproteins, ZP1, ZP2, and ZP3, all of which serve essential functions in the fertilization process (Yanagimachi, 1994). It is now clear that galactose, located in α-linkage at the non-reducing terminus of O-linked oligosaccharides is at least one of the sugars on ZP3 that imparts to it a primary role in sperm-ovum binding and subsequent triggering of the acrosome reaction (Bleil and Wassarman, 1988) while ZP2 appears to be a secondary receptor that binds spermatozoa only after induction of the acrosome reaction (Mortillo and Wassarman, 1991).

By serving as mediator and regulator of sperm-egg interactions in mammals, the ZP is a central character in the complex process that leads to fertilization of eggs by a single sperm (Aitken *et al.*, 1984; Berger *et al.*, 1989). The unusual complexity of the preliminaries to sperm-egg contact, and then of the specific interactions between the gametes, makes fertilization in mammals a potentially central point at which conception might be prevented. Contraception based on this principle appears especially attractive in light of the need to target only free cells, rather than to change the state of an organized tissue (Bedford, 1994). The realization that this process can be inhibited by specific antibodies to specific ZP antigens (Ownby and Shivers, 1972; Oikawa and Yanagimachi, 1975; Sacco and Palm, 1977; Wood *et al.*, 1981) lays the foundation to intensive subsequent study of the ZP as a source of specific target antigens for contraceptive vaccination in females.

1.2.5 The zona pellucida as a potential immunocontraceptive agent

The application of immunocontraceptive vaccines for human use demands efficacy and safety. Contraceptive vaccines are however distinct from most other vaccines in two respects. First, the target antigens are not pathogens but rather are associated with normal tissue, complicating the assessment of risk versus benefit. Second, many immunocontraceptive methods require the induction of strong immune responses against 'self' antigens. This requires that the 'self' antigen must be presented in a 'foreign' or 'non-self' form in order for the immune system to effectively respond.

Since some antigenic determinants are shared between mammalian ZP proteins (Moller *et al.,* 1990; Dunbar *et al.*, 1994) while others are species-unique (Dunbar *et al.,* 1986; Maresh and Dunbar, 1987; Skutelsky *et al.,* 1994), the problem of presentation of 'self' antigen is overcome; hetero-immunization with ZP proteins elicits the production of auto-antibodies that inhibit or reduce fertility in several species. In other words, since mammalian ZP contains species cross-reactive as well as species-unique antigenic determinants (Drell and Dunbar, 1984; Maresh and Dunbar, 1987), it is probable that the species-unique antigenic determinants ('foreign' portion) of the molecule is responsible for eliciting the immune response which results in antibody formation against cross-reactive ('self') antigens (Wood *et al.*, 1981). The immunogenicity of ZP glycoproteins is therefore primarily due to the 'foreign' epitopes associated with the ZP of different species. However, the biological effectiveness of the vaccine is provided by the antibodies directed against epitopes that are shared among different mammalian species. Of note in this regard is the pig

ZP which has attracted wide interest because it is readily obtained in quantity (Dunbar *et al.*, 1980) and because its glycoproteins are immunologically cross-reactive with those of species targeted for immunocontraception including the human (Sacco, 1977; Koyama *et al.*, 1985; Shabanowitz, 1990), non-human primates (Gulyas *et al.*, 1983; Sacco *et al.*, 1983a, 1986a), dog (Mahi-Brown *et al.*, 1982, 1985; Maresh and Dunbar, 1987), rabbit (Skinner *et al.*, 1984) and horse (Liu and Shivers, 1982; Kirkpatrick *et al.*, 1991, 1992). In addition, it has been shown that antibodies raised against the pig ZP can inhibit sperm-ZP attachment in the human (Trounson *et al.*, 1980; Koyama *et al.*, 1985; Henderson *et al.*, 1987a; Hasegawa *et al.*, 1988).

Several additional factors make the ZP antigens attractive targets for an immunocontraceptive vaccine. Firstly, despite this extensive cross reactivity between species, the antigens comprising the ZP appear to be extremely specific for this structure (Millar *et al.*, 1989; Mahi-Brown *et al.*, 1992). No similar antigens have yet been detected in any other tissue or body fluid and, as a result, the possibility of deleterious side-effects is reduced. Secondly, the ovary is not immunologically sequestered as the testis and thus its components, such as the ZP, are accessible to the humoral and cellular immune responses generated against a vaccine. Thirdly, since ZP antigens are non-circulatory (Wassarman, 1988), the risk of complications due to the formation of circulating immune complexes would appear to be minimal. Fourthly, the long period of exposure to antibodies in the follicular fluid during oocyte development means that low titres can be sufficient to decrease fertility *in vivo* (Dunbar, B. S. and Schwoebel, E. D., personal communications, 1996). Fifthly, since normally one egg is ovulated per cycle in a large number of mammalian species, a

limited number of antibodies should be required compared with the numbers needed to neutralize hormone or sperm antigens. Sixthly, the expression of ZP glycoproteins is stage-dependent during ovarian follicular development (Dunbar *et al.*, 1994). It should thus be possible to modulate the immunological effects towards the developing oocytes while maintaining at the same time normal hormonal levels. Seventhly, since antibodies against the ZP prevent fertilization (Aitken *et al.*, 1984; Skinner *et al.*, 1990), an intrinsic advantage of using the ZP as a target for the regulation of fertility is that the end result would be a block of conception rather than the induction of early abortion. Eighthly, naturally occurring autoantibodies against the ZP have been implicated in some cases of unexplained infertility (Shivers and Dunbar, 1977). Ninthly, because there are a limited number of major glycoproteins associated with the ZP (three in most species), these proteins have been well characterized (Ringuette *et al.*, 1986; Dunbar *et al.*, 1994). In addition, complementary DNA (cDNA) clones encoding two mice ZP proteins and two rabbit ZP proteins have been isolated independently (Schwoebel *et al.*, 1991; Lee *et al.*, 1993; Epifano *et al.*, 1995; Dunbar *et al.*, 1994). Finally, studies in mice (Millar *et al.*, 1989), horses (Kirkpatrick and Turner, 1991) and some primates (Sacco *et al.*, 1983a) have shown that the reversible reduction of fertility is a feasible objective.

Despite these encouraging considerations and the susceptibility of the ovum as a 'one-cell target,' there are continuing concerns as to whether ZP antigens will ultimately prove to be suitable for a contraceptive vaccine in women. Although immunization regimens involving reasonably well characterized ZP antigens can be shown to inhibit fertility effectively in the several species mentioned above, these

effects are sometimes accompanied by altered ovarian function and/or auto-immune pathology in the ovary (Wood *et al.*, 1981; Skinner *et al.*, 1984). These findings are not unduly surprising since the target antigens involved are present on developing ovarian oocytes as well as mature ova. The data also suggests that the genesis of the ovarian immunopathology cannot be adequately explained by the relative impurity of the antigenic preparations (Wood *et al.*, 1981), but that it is related to intrinsic properties of the ZP proteins (immunogens) and the type of adjuvant used (Upadhyay *et al.*, 1989; Bagavant *et al.*, 1994). While this response is preferable for the development of contraceptive and sterilization vaccines for animals, these effects are not acceptable for the development of a human contraceptive vaccine.

Bearing in mind that in some instances of ZP immunization ovarian pathology is not observed (Drell *et al.*, 1984) and that the effectiveness of the immunization depends on the species of animal being immunized and the species of ZP used as the source of immunogen (Dunbar *et al.*, 1986) or in other words the ratio of 'foreign' to 'self' determinants, it has become critical therefore, to begin to evaluate those forms of zonae pellucidae and their molecules which can be used to generate antibodies which will specifically interfere with fertilization without altering ovarian function. In addition, it is also important to evaluate various adjuvants that can be used in the design of a safe and effective human based ZP contraceptive. Thus, native rabbit ZP that is thought to cause fewer side-effects than native pig ZP (Dunbar B. S. and Schwoebel, E. D., personal communications, 1995) was tested in this study. A new adjuvant, TitreMax® was also evaluated in this study. It is further important to test these immunogens in animal models close to that of the human (as was done in this

study) because the testing of potential contraceptive vaccines is ethically unacceptable in human beings due to the possibility of negative long term effects (Wango, 1990).

1.2.6 The baboon as a model for human reproductive studies

Great apes are the closest to humans in many anatomical and physiological aspects of reproduction (Bambra, 1993). However, because all species are endangered and protected, they are not practical models for research for most studies (Bambra, 1993). There is growing evidence that the successful use of the baboon in an increasing variety of investigations justifies its status, not because it can be considered an ideal model but because it answers many of the criteria of parallelism to the human, as well as providing ease of handling, availability in large numbers, and reasonable cost. The parallelism between humans and baboons in the area of reproductive biology include hormonal control of ovarian function, general structure of the cervix, uterus, oviduct and ovary, sperm-ZP interaction and the timing and control of oogenesis and follicle selection (Hendrickx, 1971; Hausfater, 1974; Holmes, 1984; Eley and Bambra, 1993). It has a life span and development ratio to the human of 1: 3, which makes long-term studies possible. Its menstrual cycle is 28-30 days with a 27-week gestation period (Eley and Bambra, 1993) which lends this animal to studies of perinatal biology, experimental embryology and reproductive physiology.

In addition, the baboon readily accepts the captured state, and reproduces in captivity with little difficulty (Vagtborg, 1965). The female has a perineal skin that indicates the stage of the menstrual cycle with relative precision (Hendrickx, 1971; Eley and Bambra, 1993). It is also a continuous breeder and is a convenient size if

large numbers of recurrent blood samples are required, or complex experimental surgery is to be conducted (Bambra, 1993). The baboon is a social animal and can be studied from anthropological, social and ecological standpoints (Vagtborg, 1965). These factors make it an excellent model for the evaluation of the efficacy of contraceptive vaccines as well as in the identification of potential adverse long-term side-effects that may not be observed in animals of shorter life-spans.

1.2.7 Project aims

The overall aim of this project was therefore to perform a comparative evaluation, in terms of immunogenicity and ovarian effects, between native rabbit ZP and native pig ZP immunization in the female baboon using TitreMax® as adjuvant. The specific aims arising from the overall aim were as follows.

1). To determine if antibodies are produced in response to immunization with native rabbit ZP or native pig ZP in a non-human primate and if so, which one produces higher and longer sustained titres.

2). To evaluate whether the antibodies produced can recognize the respective immunogen in addition to bacterially produced recombinant protein encoding the rabbit ZP antigen.

3). To determine if native rabbit ZP or pig ZP immunization can cause alteration in ovarian function using a species that can easily be evaluated for ovarian cyclicity.

4). To determine whether the antibodies produced can also recognize native baboon ZP and hence ascertain the presence of autoantibodies.

5). To evaluate the efficacy of a synthetic adjuvant in a non-human primate.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Time, place and animal of study

This experimental study was conducted for a period of 12 months at the Institute of Primate Research which is located 25 kilometers south of Nairobi. For this study, the baboon (species *Papio anubis* or olive baboon), which is an Old World Monkey belonging to the Anthropoidea sub-order of non-human primates, was used. This species is found over a wide range in Africa and is a large primate that is especially desirable for surgery and reproductive physiology research (Holmes, 1984).

2.1.2 Antigens for immunization

The native rabbit and pig ZP used for this study were kindly donated by Professor B. S. Dunbar of Baylor College of Medicine, Houston, Texas, USA. The antigens were of 99% purity.

2.1.3 Adjuvant for immunization

TiterMax® was the adjuvant used and it was obtained from Vaxcel™, Incorporated, 3000 Northwoods Parkway, suite 200, Norcross, Georgia 30071, USA. This adjuvant is formulated with squalene, a metabolizable oil and the active ingredient is a synthetic block copolymer, CRL89-41, bonded to silica particles. These copolymer-coated microparticles act as a stabilizer for the water-in oil emulsion. TiterMax® also contains a surfactant. Good immune responses have been achieved with ratios of water to TiterMax® of 50 to 90%, but the 50: 50 water-in oil emulsion (as was used in this case) is usually optimal. This adjuvant is stored at 4 °C.

2.2 Methods

2.2.1 Sample size determination

For studies where the aim is to demonstrate a significant difference in the means of two groups (such as this one), the formula for calculation of sample size is as follows:

$$n = (U+V)^2(\delta_1^2 + \delta_2^2)$$

$$(\mu_1 - \mu_2)$$

where,

n	=	sample size in each group,
U	=	one-sided percentage point of the normal distribution corresponding to 100%- the power,
V	=	percentage point of the normal distribution corresponding to the two-sided significance level,
δ_1, δ_2	=	standard deviations of the two groups and
$\mu_1 - \mu_2$	=	difference between the means.

Since native rabbit ZP has never been tested before in the female baboon, this formula could not be used. The literature, however, revealed that four animals for each group was an adequate sample size (Wood *et al.*, 1981; Paterson *et al.*, 1992; Prasad *et al.*, 1995; VandeVoort *et al.*, 1995).

2.2.2 Animal husbandry and evaluation of ovarian cyclicity

Eleven cycling female baboons were used in this study. After capture, quarantine and identification by tattooing (on the inner aspect of the left thigh) they were housed in two group cages. The cages provided 3 m^2 per occupant to prevent overcrowding. The baboons were fed with monkey cubes from Unga Limited, Kenya (at 35 g/kg body weight) at 9:30 a.m. every day and with fruit and vegetables at 3:00 p.m. three times per week. Ascorbic acid powder was sprinkled on the monkey chow daily during feeding; primates cannot synthesize vitamin C and therefore require an external source. Water was provided 'ad libitum.' The above feeding schedule, in the experience of IPR researchers, has proved to be adequate for health and maintenance of baboons (Suleman, 1993). Cleanliness of the cages and health of the baboons was maintained at all times. During all experimental procedures, the animals were treated humanely in accordance with the IPR guidelines for the care and use of laboratory animals.

Assessment of ovarian cyclicity was done by sex skin evaluation which is an accepted general way to stage the menstrual cycle in the baboon (Hendrickx, 1971; Eley and Bambra, 1993) since dramatic changes in colour and size occur throughout the cycle. The degree of inflation or tumescence in any individual animal indicates the stage of the cycle that she is in. Monitoring of all females was done every day and a number assigned to the degree of inflation using the method of Eley and Bambra (1993) as described in Appendix 1.

2.2.3 Immunization of baboons with native pig and rabbit zona pellucida antigens, and blood collection

The eleven cycling female baboons were randomly assigned (using the table of random numbers) into three groups comprising three controls and four each for the treatment groups rabbit ZP and pig ZP.

2.2.3.1 Solubilization of zona pellucida antigens

The antigens, obtained in lyophilized form, were solubilized using a 0.1M sodium carbonate buffer, pH 9.6 (Appendix 2) as follows. After adding the appropriate amount of solubilization buffer to the tubes containing the antigens, the tubes were placed in a pre-warmed water bath (at 60 °C) [Thermolyne Dri-Bath type 16500, USA] for approximately 10 minutes. After every 2 minutes, the solutions were pipetted in and out severally to facilitate solubilization.

2.2.3.2 Emulsification and injection of antigens

TiterMax® adjuvant was used. This adjuvant and antigens for immunization were emulsified as described in Appendix 6. The emulsion was then injected, using different needles (21 gauge) [Becton Dickinson, USA], into each animal. All immunizations were given by subcutaneous injection dorsally between the scapulae in two loci at different sites at each subsequent immunization. To facilitate handling, the animals were sedated with 10 mg/kg of Ketamine hydrochloride (Bayer, East Africa). Asepsis was assured by Hibitane® soaked cotton swabs. Three immunizations, 21-days apart, were performed on each animal. At every immunization, the animals received a total of 200 μl each of immunogen that contained 150 μg of ZP proteins. The 200 μl of injection solution for the control animals contained solubilization buffer

and adjuvant only that were emulsified in a similar manner. Before each immunization, and periodically thereafter, blood samples were collected from the femoral vein for antibody analysis. The immunization and blood collection schedule is summarized in Table 2 below.

Table 2 Immunization and blood collection schedule

Prim	Bst 1	Bst 2	3 wk bleed	Bl 1	Bl 2	Bl 3	Bl 4	Rest
D0	D21	D42	D62	D76	D91	D106	D121	Monthly
30 ml	30 ml	30 ml	30 ml	20 ml	20 ml	20 ml	20 ml	30 ml

Key

Prim : Primary immunization

Bst 1, Bst 2 : First and second boosts respectively

3 wk bleed : Bleed taken three weeks after the last boost

Bl 1-4 : Four bleeds taken two weeks apart

D : Days of study with day of primary immunization taken as day 0

20 ml, 30 ml : Quantities of blood collected from each animal

Note that due to unexpected limitations in the amount of native antigen available, two animals of the rabbit ZP group received only one boost while the other two received the required two boosts.

2.2.4 Evaluation of antibody titres by enzyme-linked immunosorbent assay (ELISA)

The following procedure adapted from Maresh and Dunbar (1987) was used for this assay. Micotitre titre plates (96-well Immulon 2 U, Dynatech, USA) were coated with antigen, diluted in antigen coating buffer, at a concentration of 1 ηg/µl. Controls for each assay included; no antigen adsorbed to wells, no primary antibody, non-immune serum (or immune sera from animals injected with adjuvant alone) and no secondary antibody. 50 µl of antigen (in antigen coating buffer) was added to each microtitre plate well. The plates were then incubated at room temperature (25 °C) for 6 hours, while shaking using Mistral™ Magnestir® apparatus (Lab-line Instruments, USA).

After incubation, the contents were removed and the plates were thoroughly washed four times with assay buffer. After each wash, the contents were shaken out sharply and thoroughly to prevent mixing of reagents between wells. The plates were then blocked by adding 100 µl/well of blocking solution. This was followed by overnight incubation, with shaking, at 4 °C. After the incubation, the plates were again washed four times with assay buffer. Primary antibody dilutions (from sera of experimental animals) were then applied to microtitre wells (50 µl/well) followed by overnight incubation, with shaking, at 4 °C. The antibody dilutions were then removed from the wells and the plates washed routinely with assay buffer. Care was again taken to ensure reagents did not mix between wells during the washes. Inactivating reagent (100 µl/well) was then added followed by incubation at room temperature (25 °C) with shaking for 2 hours. After incubation, plates were routinely

washed and secondary antibody (peroxidase-conjugated rabbit IgG fraction to human IgG Fc, Organon Teknika, USA) dilutions (50 µl/well) were then added. This was followed by overnight incubation at 4 °C with shaking. Again, plates were routinely washed after the incubation and o-Phenylenediamine dihydrochloride (OPD) [Sigma, USA] substrate solution (50 µl/well) added. The plates were incubated for 30 minutes at room temperature with shaking. The reaction was stopped with dilute acid solution after which the microtitre plates were read using a microtitre ELISA reader (Dynatech, USA) at 450 ηm absorbance.

These ELISA experiments were done in duplicate in three separate experiments to establish the point at which 50% maximum binding was observed. Baboon anti-TitreMax® adjuvant was used as control to determine the amount of nonspecific binding, which was then subtracted from the antibody binding data. Methods of preparation of buffers, reagents, solutions and substrate used for these experiments are contained in Appendix 3.

2.2.5 Antibody detection and analysis by 1D-PAGE immunoblotting

This procedure was done to determine whether the immunization resulted in the production of antibodies not only against the immunogen (native pig ZP or rabbit ZP) but also against bacterially produced recombinant (BPR) rabbit ZP protein. The recombinant proteins used in this study were kindly donated by Dr. E. D. Schwoebel, of the Institute of Primate Research, Nairobi, Kenya, who, together with Lee and others, had previously cloned and characterized cDNAs encoding these proteins (Schwoebel *et al.*, 1991, 1992; Lee *et al.*, 1993). These recombinant proteins were expressed in pEX bacterial expression vectors as temperature-inducible

cro-β-galactosidase fusion proteins.

Low molecular weight markers (BioRad, USA) together with protein from native rabbit and pig ZP and the recombinants underwent one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1D SDS-PAGE) according to the following procedure of Dunbar (1987). Methods of preparation of buffers, reagents, solutions and substrate used for immunoblotting are contained in Appendix 4.

2.2.5.1 Preparation of samples for 1D SDS-PAGE

Solubilization buffer was added to protein samples (protein concentration at 0.05 μg/μl). These mixtures were then heated for 5-10 minutes at 95 °C in a boiling water bath using plastic eppendorf® tubes (Merck, Germany). Caution was taken to ensure that the solutions did not evaporate during heating.

2.2.5.2 Procedure for casting running gel

Clean dry glass plates and spacers (BioRad, USA) were assembled. Bis-acrylamide (BioRad, USA), running gel buffer and water were then combined in a glass beaker. N, N, N', N'- Tetramethylenediamine (TEMED) [BioRad, USA] was then added and the solutions mixed by thoroughly swirling the beaker. 10% ammonium persulphate (APS) [BioRad, USA] was then added followed by gentle swirling to facilitate mixing. Depending on the relative amounts of these solutions, various percentages of running gels can be made (Table 8). 7.5% gels were routinely found to give the best results for this assay. The mixture constituting 7.5% gel was then poured down one edge of the spacer of the gel casting unit using a 10-ml glass pipette. This mixture which was filled to within 4 cm of the top of the glass plates was

carefully overlaid with water saturated sec-butanol (Sigma, USA) and allowed to polymerize for 45-60 minutes. When the running gel was polymerized, the water saturated sec-butanol was removed and the gel rinsed several times with distilled water, and drained well.

2.2.5.3 Procedure for casting stacking gel, sample loading and electrophoresis

The same stacking gel was used regardless of the percentage acrylamide in the running gel. After combining stacking gel buffer, bis-acrylamide (BioRad, USA) and distilled water, TEMED (BioRad, USA) was added and mixed by swirling. Ammonium persulphate (BioRad, USA) was then added and the solution swirled to facilitate mixing. This solution was then layered on top of the running gel. A plastic comb (BioRad, USA) was inserted into the stacking gel to form wells. Caution was taken to ensure that no bubbles were trapped on the bottom of the comb dividers. Tank buffer was then prepared and the gel assembled in the tank chamber. After the gel plate became attached to the upper buffer reservoir, samples were underlain in wells using a Hamilton® syringe (Joh.Achelis and Johne, Germany). Electrophoresis was then carried out (using model 1000/500 electrophoresis unit, BioRad, USA for power supply) at 167 volts until the dye front reached the bottom of the gel.

2.2.5.4 Transfer to nitrocellulose paper and probing with antibodies

The unstained, unfixed 1D-PAGE gel was then placed on the cathode side of nitrocellulose paper (BioRad, USA). Transfer was carried out for 15 minutes at 0.03 amps using the E-C Electroblot apparatus transfer unit (BioRad, USA). The unstained nitrocellulose paper (after transfer) was blocked for 2 hours, on a shaking platform (model ika-vibrax-VXR, Joh.Achelis and Johne, Germany), at 25 °C in one times

(1X) phosphate buffered saline (PBS), pH 7.3 with 5% (weight/volume) non-fat powdered milk (KCC, Kenya). This was followed by overnight probing at 4 °C, on a rocking platform (Joh.Achelis and Johne, Germany), in 5% non-fat powdered milk with antibody (from serum of experimental animals, diluted 1: 25). After probing, the antibody solution was removed and the paper washed five times (5 minutes each while shaking) in 5% non-fat powdered milk in 1X PBS. This was followed by probing with secondary antibody (peroxidase conjugated rabbit IgG to human IgG or peroxidase conjugated rabbit IgG to Cynomolgus monkey IgG, both from Organon Teknika, USA)) diluted 1: 500 in 5% non-fat powdered milk in 1X PBS. This probing was carried out for 6 hours at room temperature (25 °C) while rocking. The antibody solution was then removed and the paper washed five times with 1X PBS while shaking. 3'3-Diaminobenzidine tetrahydrochloride (DAB) [Sigma, USA] substrate solution was then added and the reaction allowed to develop for 2-10 minutes. The reaction was then stopped by washing with 1X PBS for 5 minutes. The nitrocellulose paper was then allowed to dry at room temperature (25 °C) while protected from light. Photographs were taken after drying was complete.

2.2.6 Immunohistochemistry for antibody detection and analysis

This procedure was done to evaluate whether the immunization resulted in antibodies that can recognize the ZP of the animal (baboon) under test. Previously collected baboon ovaries from non-experimental animals were used. These ovaries had previously been subjected to the same histological procedure as above up to the sectioning level. The tissue sections (5 μm) were then mounted on frost ended slides (Chance Propper, England) that had previously been washed as follows; washing in

0.2 M hydrochloric acid (Kobian, Kenya) for 20 minutes, rinsing 5 times in tap water, rinsing 5 times in distilled water and then dipping in 100% ethanol (Sigma, USA) for 1 minute. The tissue-mounted slides were then placed in an oven at 42 °C for overnight incubation. After placing the slides in a staining rack, they underwent the following procedure (modified from Vectastain® ABC kit, USA; instructions for immunohistochemical staining).

Deparafinization and rehydration- done by passing the tissue sections in the following solutions; 2 changes of xylene (Sigma, USA) for 6 minutes each, 100% ethanol (Sigma, USA) for 6 minutes, 2 changes of 95% ethanol for 6 minutes each, 85% ethanol for 6 minutes, 70% ethanol for 6 minutes and 50% ethanol for 6 minutes. Endogenous peroxidases were then inactivated using a mixture of 99.7% methanol (Sigma, USA) and 0.3% hydrogen peroxide (Sigma, USA) for 20 minutes. This was followed by washing in 1X PBS 3 times for 5 minutes each. Blocking of non-specific binding was achieved using a 1: 50 dilution of normal rabbit serum in 3% bovine serum albumin (BSA) [Sigma, USA] in 1X PBS, 100 μl per section for 4 hours at room temperature. The tissues were then probed overnight at 4 °C with primary antibody (from serum of experimental animals) diluted 1: 10 in 3% BSA in 1X PBS, 100 μl per section. This was followed by washing 3 times for 5 minutes each with 0.1% BSA in 1X PBS. After this, the tissues were then probed for 5 hours at room temperature with secondary antibody (peroxidase conjugated rabbit IgG fraction to human IgG Fc, Organon Teknika, USA) diluted 1: 200 in 3% BSA in 1X PBS, 100 μl per section. The tissues were then washed 3 times for 5 minutes each with 0.1% BSA in 1X PBS followed by a 5-minute wash in 1X PBS alone. DAB

substrate solution was then liberally added onto the tissue sections and 2-10 minutes allowed for colour change. The reaction was then stopped by dipping the slides (with the tissue sections) in 1X PBS. The sections were then passed backwards through the rehydration and deparafinization process above. Finally, the tissues were mounted using permount.

Note that for this experiment rabbit anti-pig ZP antibody was used as a positive control with the secondary antibody being whole molecule peroxidase conjugated goat anti-rabbit IgG (Sigma, USA). For all the incubations the tissue sections were placed in a humid chamber. Throughout the whole procedure caution was taken to ensure that the tissue sections did not dry. Methods of preparation of buffers, solutions and substrate for these immunohistochemical experiments are contained in Appendix 5.

2.2.7 Ovariectomies and preparation of ovaries for histological analysis

Unilateral ovariectomy was performed periodically in one animal of each group beginning 50 days after the first immunization as outlined in Table 3 below.

Table 3 Schedule for left unilateral ovariectomies.

Animal number	Treatment group	Day of ovariectomy *	Stage of MC
1994	Pig ZP	50	0
2032	Pig ZP	61	6
2016	Control	121	2
2020	Pig ZP	121	1
1991	Rabbit ZP	183	6
2001‡	Rabbit ZP	183	2
2033	Control	273	2
2044	Pig ZP	273	6
1992‡	Rabbit ZP	275	4
1997	Rabbit ZP	275	6
2025	Control	365	0
2020	Pig ZP	365**	4

Key

* : Days counted from primary immunization with this day taken as day 0

** : Terminal point for this animal whose right ovary was also removed on day 365

‡ : Animals in rabbit ZP treatment group that received only one instead of two boosts of immunogen

MC : Menstrual cycle (see Appendix 1)

Pig ZP : Immunized with pig zona pellucida

Rabbit ZP : Immunized with rabbit zona pellucida

For the surgeries the animals were induced with a mixture of Ketamine hydrochloride at 10 mg/kg and Xylazine hydrochloride at 10 mg/kg; both from Bayer East Africa. They were maintained with a mixture of Halothane, nitrous oxide and oxygen using a paediatric anaesthetic machine. Catheterization of the urethra was done to drain the urine from the bladder. After ensuring asepsis, a 2-inch incision was made over the bladder and the left ovary removed. Closure of the abdominal incision was done routinely.

After removal, the ovaries were divided into two, labeled and processed for histology using the following procedure. The halves were divided into blocks 2 mm^3 in size. Blocks from one half were then fixed in Bouin's solution (Sigma, USA) for 8 hours at room temperature (25 °C) and those from the other half in 10% buffered formalin (Sigma, USA) for 16 hours also at room temperature. After fixation, the blocks of tissue were dehydrated in 50% ethanol 3 times for a period of 20 minutes each. The tissues were then passed through similar dehydrations in increasing strengths of ethanol (70%, 85%, 95%, and 100%) followed by two immersions in xylene for 6 minutes each. The tissue blocks were then embedded in paraffin wax. For histological evaluation, the tissue blocks were serially sectioned (7 μm thickness) using a microtome (Leitz, model 1512, Germany). The sections were routinely stained with Haematoxylin and Eosin for microscopic observation.

2.2.8 Histological analysis

Using light microscopy (Leitz Dialux, model 22 EB, Germany) techniques coupled with morphometric analysis, the presence, type and status of developing follicles were assessed in ten different cross-sections taken at different regions of the ovary and representative photomicrographs (model U-ACAD, Olympus BX 50, Optical Co., Japan) taken . Sections in which large corpora lutea constituted the majority of the ovary were excluded from analysis. To exclude bias, the histologist was blinded with respect to the origin of the ovarian sections.

Using the method of Baker (1987) the developing follicles were subdivided into the following types or stages.

i) Primordial follicles - oocytes surrounded by a single layer of flattened epithelial cells.

ii) Primary follicles - oocytes surrounded by a single layer of cuboidal granulosa cells.

iii) Secondary follicles - oocytes surrounded by more than one layer of cuboidal granulosa cells with no evidence of antral formation.

iv) Tertiary follicles - antral formation evident.

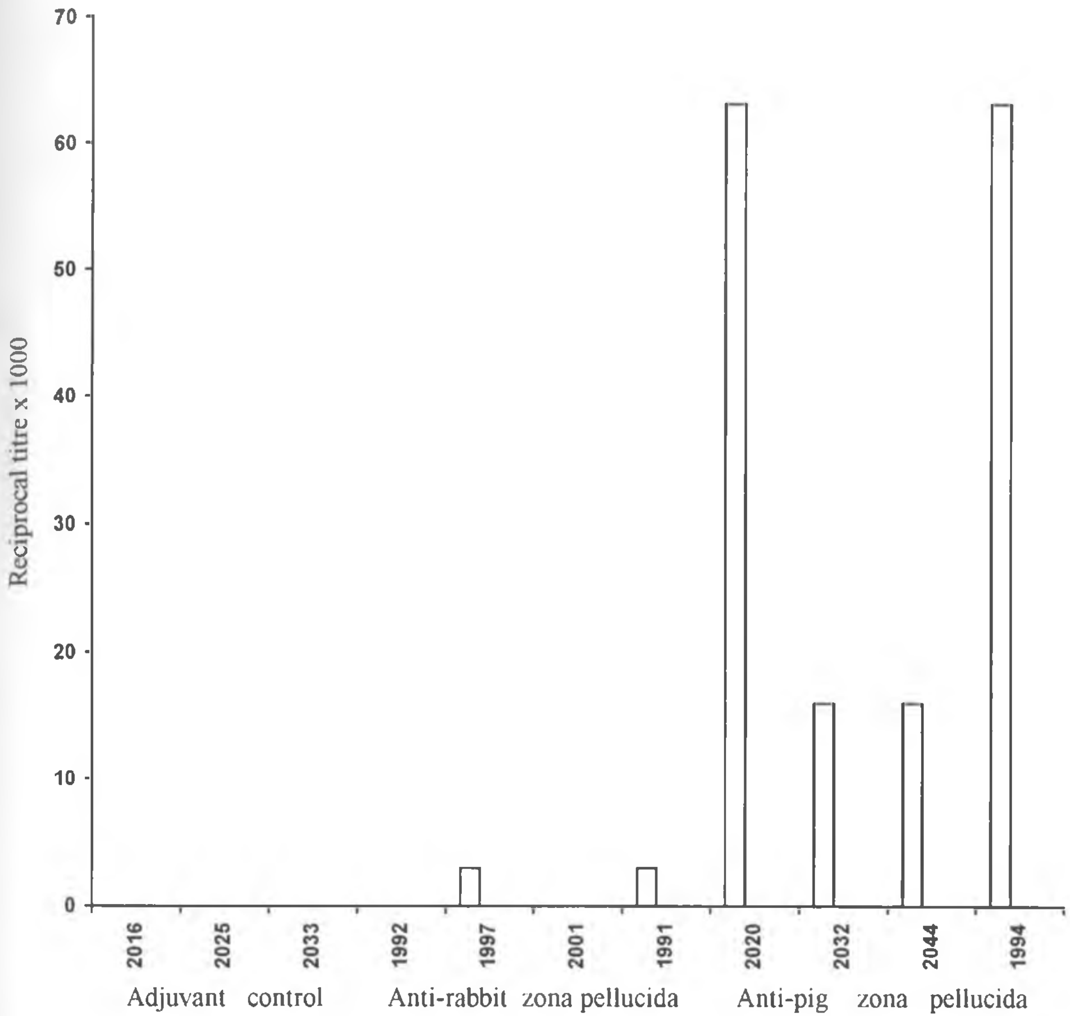
The results of the experimental groups (pig and rabbit ZP) were compared with that of the control group and with each other for each time frame and across the study period.

CHAPTER 3. RESULTS

3.1 Antibody titres as determined by enzyme-linked immunosrbent assay

Due to lack of adequate native antigen for coating of ELISA plates and problems routinely associated with standardization of ELISA on ZP antigen, the sera from each animal's bleeds for the first 5 months (post-immunization) of the study were pooled and this pool used for the ELISA assay. It was presumed that after the first 5 months, using TitreMax® as adjuvant, the antibody titres would be negligible (Dunbar, B. S., unpublished communications, 1996). The results of the ELISA assay for all the groups of animals are presented in Figures 1 and 2 below.

Figure 1 ELISA assay on pig zona pellucida



Key

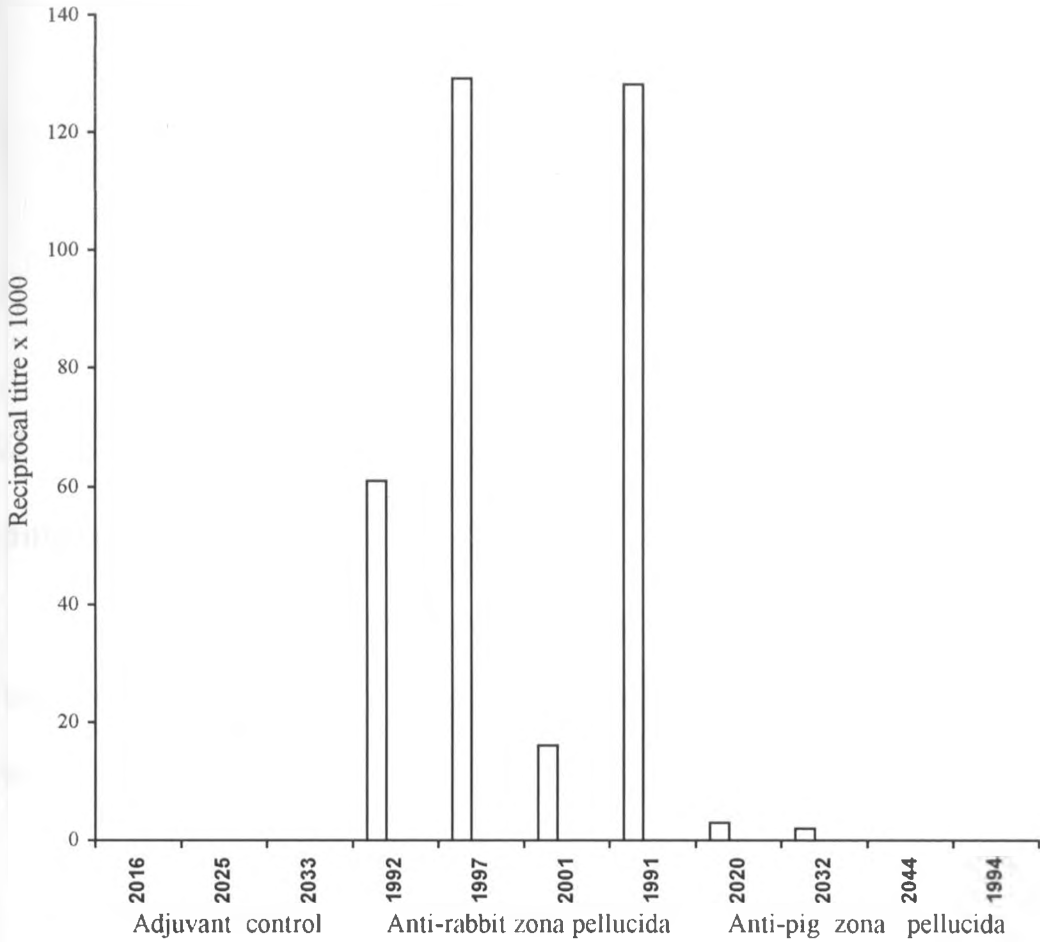
Adjuvant control : Immunized with adjuvant alone

Anti-rabbit zona pellucida : Immunized with rabbit zona pellucida

Anti-pig zona pellucida : Immunized with pig zona pellucida

Note that animal numbers 1992 and 2001 received only one boost instead of the required two boosts of immunogen.

Figure 2 ELISA assay on rabbit zona pellucida



Key

Adjuvant control : Immunized with adjuvant alone

Anti-rabbit zona pellucida : Immunized with rabbit zona pellucida

Anti-pig zona pellucida : Immunized with pig zona pellucida

Note that animal numbers 1992 and 2001 received only one boost instead of the required two boosts of immunogen.

The ELISA assay demonstrated the following.

1) Both native heat solubilized pig and rabbit ZP induced antibody production in female baboons using TitreMax® as adjuvant.

2) All four baboon anti-pig ZP sera recognized native pig ZP but only two (animal numbers 2020 and 2032) had low titres against native rabbit ZP.

3) All four baboon anti-rabbit ZP sera recognized native rabbit ZP but only two (animal numbers 1991 and 1997) had low titres against native pig ZP.

3.2 Characterization of induced antibodies by 1D-PAGE immunoblotting

Figures 3 and 4 below show the immunoblot patterns of representative animals from each group while Table 4 below summarizes the immunoblot results for all the animals in the study.

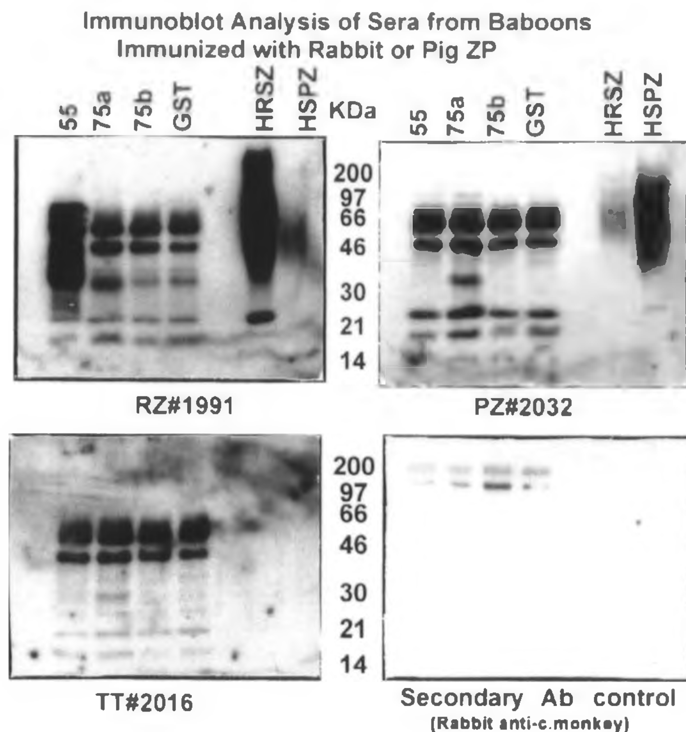


Figure 3 1D-PAGE immunoblot patterns (using rabbit IgG to Cynomolgus monkey IgG as secondary antibody) of representative animals from each group

Legend

ZP	: Zona pellucida
55, 75a and 75b	: Molecular weights of portions of bacterially produced recombinant rabbit zona pellucida antigen with 75a and 75b representing portions of the 75-kDa protein
GST	: Control lane for recombinant proteins
HRSZ	: Heat solubilized rabbit zona pellucida
HSPZ	: Heat solubilized pig zona pellucida
KDa	: Kilodalton
RZ#1991	: Animal number 1991; immunized with rabbit zona pellucida
PZ#2032	: Animal number 2032; immunized with pig zona pellucida
TT#2016	: Animal number 2016; immunized with adjuvant only

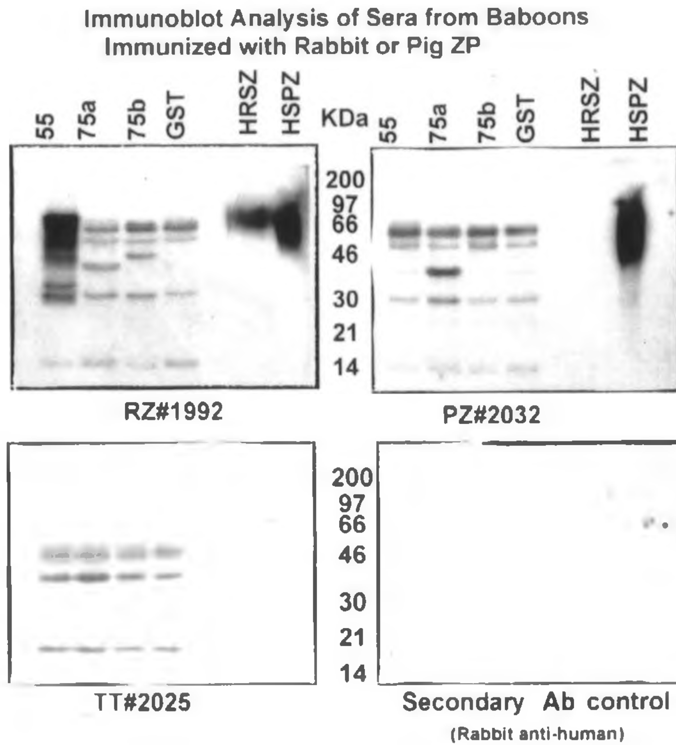


Figure 4 1D-PAGE immunoblot patterns (using rabbit IgG to human IgG as secondary antibody) of representative animals from each group

Legend

ZP	: Zona pellucida
55, 75a and 75b	: Molecular weights of portions of bacterially produced recombinant rabbit zona pellucida antigen with 75a and 75b representing portions of the 75-kDa protein
GST	: Control lane for recombinant proteins
HRSZ	: Heat solubilized rabbit zona pellucida
HSPZ	: Heat solubilized pig zona pellucida
KDa	: Kilodalton
RZ#1992	: Animal number 1992; immunized with rabbit zona pellucida (got one boost instead of the required two)
PZ#2032	: Animal number 2032; immunized with pig zona pellucida
TT#2025	: Animal number 2025; immunized with adjuvant only

Table 4 Immunoblot recognition of zona pellucida antigens by immune sera

	Native pig ZP	Native rabbit ZP	75a	75b	55
2016*	-	-	-	-	-
2025*	-	-	-	-	-
2033*	-	-	-	-	-
1991†	+	+	+	+	+
1992†	+	+	+	+	+
1997†	+	+	+	+	+
2001†	+	+	+	+	+
1994‡	+	+	+	-	-
2020‡	+	+	+	-	-
2032‡	+	+	+	-	-
2044‡	+	+	+	-	-

Key

* : Control animals

† : Rabbit zona pellucida-immunized animals

‡ : Pig zona pellucida-immunized animals

+ : Recognition

- : Non-recognition

75a, 75b and 55 : Molecular weights of portions of bacterially produced recombinant rabbit ZP antigen with 75a and 75b representing portions of the 75-kDa protein

Note that animal numbers 1992 and 2001 received only one boost instead of the required two boosts of immunogen.

The 1D-PAGE immunoblot patterns verified the ELISA results in that the antibodies in the sera of individuals of both groups of ZP-immunized animals recognized their respective immunogen and also the cross-reactive immunogen. In addition, the sera also recognized bacterially-produced recombinant protein (BPRP) encoding portions of the rabbit ZP protein. Recognition of the recombinants was very specific; all baboon anti-pig ZP sera recognized the 75a portion only while all baboon anti-rabbit ZP sera recognized all the recombinants under test. Sera obtained 3 weeks after the last boost of immunogen gave the best results for the ZP-immunized animals.

It should be noted that the sensitivity of this assay depended also on the type of secondary antibody used. Rabbit IgG to Cynomolgus monkey IgG gave recognition of native rabbit ZP by immune sera from the pig ZP group while rabbit IgG to human IgG did not. The former secondary antibody also resulted in sharper signals on immunoblot analysis than the latter secondary antibody. On the other hand, for the rabbit ZP group, rabbit IgG to human IgG gave a clearer delineation between the recombinant 75-kDa peptides and the GST control than did rabbit IgG to Cynomolgus monkey IgG.

3.3 Immunohistochemical evaluation of induced antibodies

Sera from all animals in the experiment were used to check for the presence of antibodies that were capable of recognizing baboon ZP. Figures 5, 6, 7 and 8 below are immunohistochemical sections of normal baboon ovaries probed with immune sera from rabbit anti-pig ZP (positive control) and representative animals from the three groups.

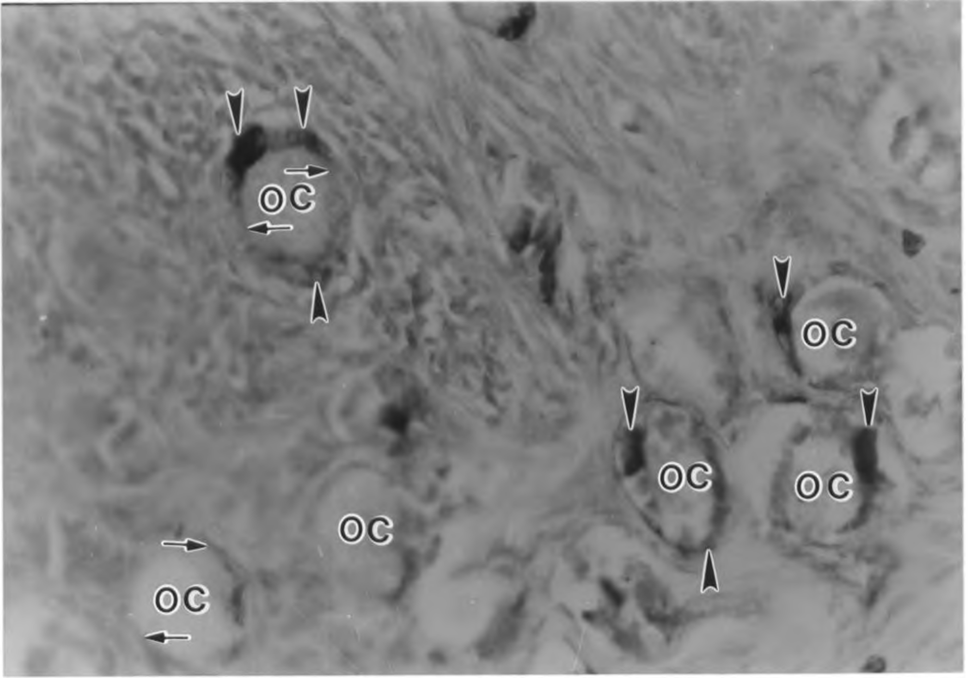


Figure 5 Immunohistochemical section of normal baboon ovary probed with rabbit anti-pig zona pellucida (positive control), and counterstained with Haematoxylin, showing positive staining of the zona pellucida

Magnification X 200; Filter LBD

Legend

Arrow	: Zona pellucida
Arrow head	: Granulosa cell
OC	: Oocyte cytoplasm

Note that almost all the zonae pellucidae and adjacent layer of granulosa cells have been stained. In addition, there is slight staining of oocyte cytoplasm.

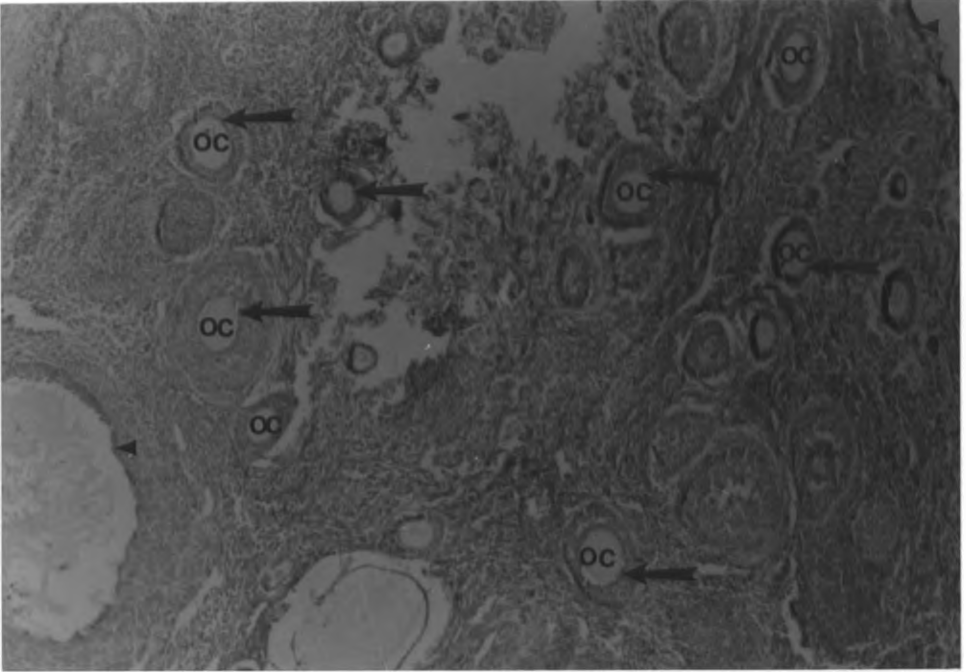


Figure 6 Immunohistochemical section of normal baboon ovary probed with immune serum from 2025 (control group) and counterstained with Haematoxylin, showing negative staining of the zona pellucida

Magnification X 40; Filter LBD

Legend

Arrow	: Zona pellucida
Arrow head	: Non-specific staining
OC	: Oocyte cytoplasm

Note that neither the oocyte cytoplasm, zona pellucida nor granulosa cells have been stained.

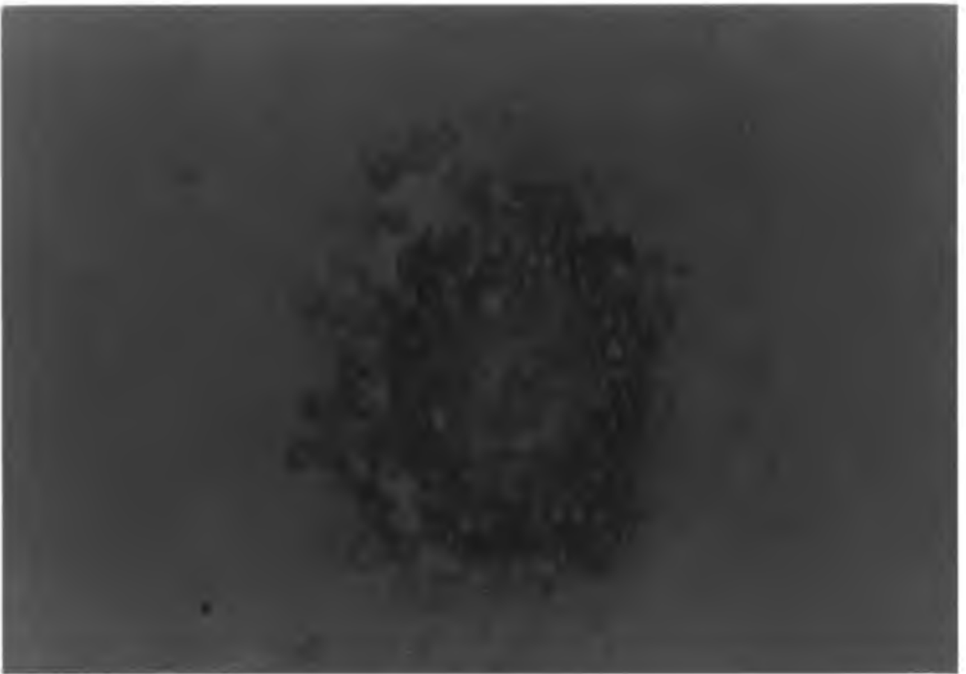


Figure 7 Immunohistochemical section of normal baboon ovary probed with immune serum from 2020 (pig ZP group), showing positive staining of the zona pellucida

Magnification X 200; Filter LBD

Legend

Arrow	: Zona pellucida
Arrow head	: Granulosa cell
OC	: Oocyte cytoplasm

Note the deep staining of the oocyte cytoplasm and granulosa cells. The zona pellucida is not clearly delineated.

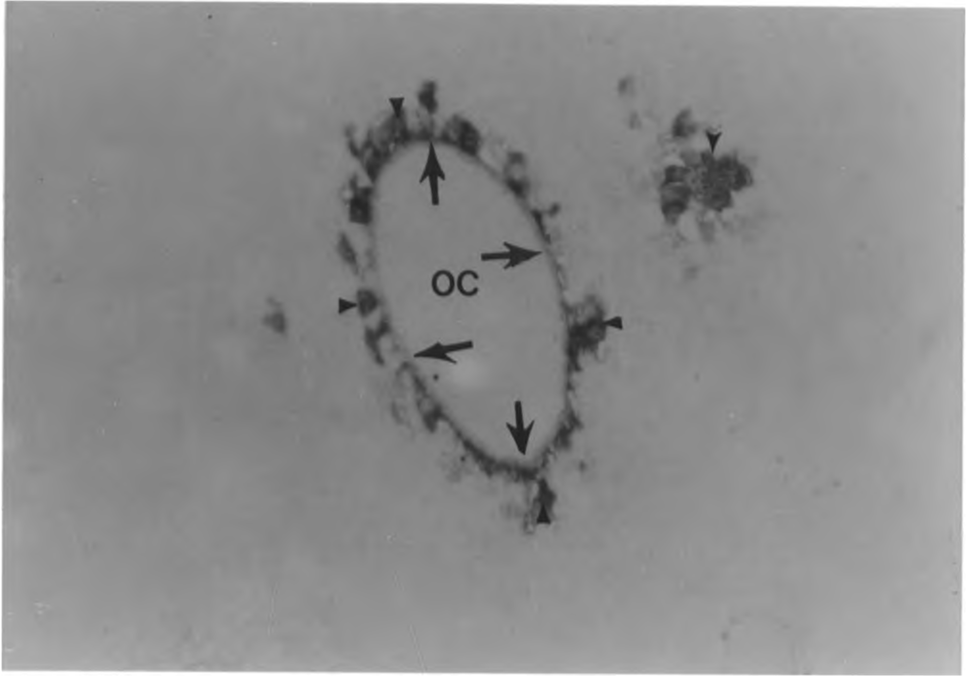


Figure 8 Immunohistochemical section of normal baboon ovary probed with immune serum from 1991 (rabbit ZP group), showing positive staining of the zona pellucida

Magnification X 200; Filter LBD

Legend

Arrow	: Zona pellucida
Arrow head	: Granulosa cell
OC	: Oocyte cytoplasm

Note the clear delineation and deep staining of the zona pellucida, adjacent granulosa cells and the very light staining of the oocyte cytoplasm.

Sera from all the ZP-immunized animals at 1:10 dilution gave a positive signal with the rabbit ZP group giving a more positive signal. Sera obtained 3 weeks after the last boost of immunogen gave the best results for the ZP-immunized animals. In addition, the immune sera of the two rabbit ZP animals (numbers 1992 and 2001) that received only one boost of immunogen instead of the required two boosts, gave a positive immunohistochemical signal. On the other hand, comparable immune sera from the pig ZP group (sera after the first boost) resulted in a negative immunohistochemical signal. All the sera from the control animals gave a negative signal.

These results show that immunization of female baboons with either native pig ZP or rabbit ZP resulted in antibody populations that immunohistochemically recognized native baboon ZP.

3.4 Gross morphology of ovaries

Left unilateral ovariectomy was performed on each animal and Table 5 below summarizes the gross morphological findings.

Table 5 Gross morphology of ovaries

Animal #	*Day of removal	Weight of ovary (g)	**Dimensions	Significant findings
1994 (PZP)	50	0.4	1.0 x 0.9 x 0.8	CL
2032 (PZP)	61	0.6	0.9 x 0.8 x 0.7	CL
2016 (C)	121	0.8	0.8 x 0.7 x 1.0	None
2020 (PZP)	121	0.6	1.1 x 0.8 x 0.8	Cystic
1991 (RZP)	183	0.9	1.3 x 0.8 x 1.2	Very big protruding CH Receded CL
2001 (RZP)∫	183	0.5	1.2 x 0.8 x 1.0	Protruding CL
2033 (C)	273	0.7	1.2 x 0.5 x 0.9	None
2044 (PZP)	273	1.0	1.4 x 0.8 x 1.1	Big CH
1992 (RZP)∫	275	0.6	1.2 x 1.0 x 0.8	Big CH Cystic
1997 (RZP)	275	1.4	1.8 x 1.2 x 1.1	Cystic (about one-third the volume of the ovary)
2025 (C)	365	1.0	1.5 x 1.3 x 1.0	Receding CH CL found to cover about one half of cut surface.
2020 (PZP) ***Terminal point	365	1.8	1.9 x 1.5 x 1.3	Transparent cyst protruding from the surface of the ovary. On sectioning cyst found to occupy about one half the volume of the ovary.

Key

: Number

∫ : Received only one boost of immunogen instead of the required two

*	: Day of removal is dated from the day of primary immunization with this day being taken as day 0
**	: Dimensions are length x breadth x width and are in centimetres
***	: Terminal point for this animal as this was the second ovary being removed
CL	: Corpus luteum
CH	: Corpus hemmorhagicum
C	: Control animal
g	: Grams
PZP	: Pig zona pellucida immunized animals
RZP	: Rabbit zona pellucida immunized animals

Table 5 above shows that immunization with the ZP antigens probably affected the gross anatomy of the ovaries as shown by the presence of cysts in some of the ovaries of the ZP-immunized animals. It should be noted that only one animal of the pig ZP-immunized group (number 2020) had cystic ovaries. On the other hand, two animals of the rabbit ZP-immunized group had ovarian cysts. Suprisingly, one of them (number 1992) had received only one boost of immunogen instead of the required two. At the endpoint of the experiment, the rabbit ZP group had not reached 365 days and so comparison for this group was limited to 275 days. The control group had no cystic ovary at all. It should be noted that the significance of these cysts could not be statistically analyzed as the sample size (one per group for each time period) was not adequate. In addition, structures associated with normal cyclical ovaries such

as corpora lutea, corpora hemmorhagica and macroscopic follicles were found in all animals of each group throughout the study period.

3.5 Histological analysis of ovaries

A minimum of 10 cross sections (7 μm in thickness) taken from different regions of the ovary of each animal were evaluated for developing follicles. In the instances where large developed corpora lutea were the majority of the section, these sections were not used to identify follicles. The presence and morphology of follicles of different stages is summarized in Table 6 below. Typical histomorphology of ovaries of animals from each of the three groups are shown in Figures 9, 10 and 11.

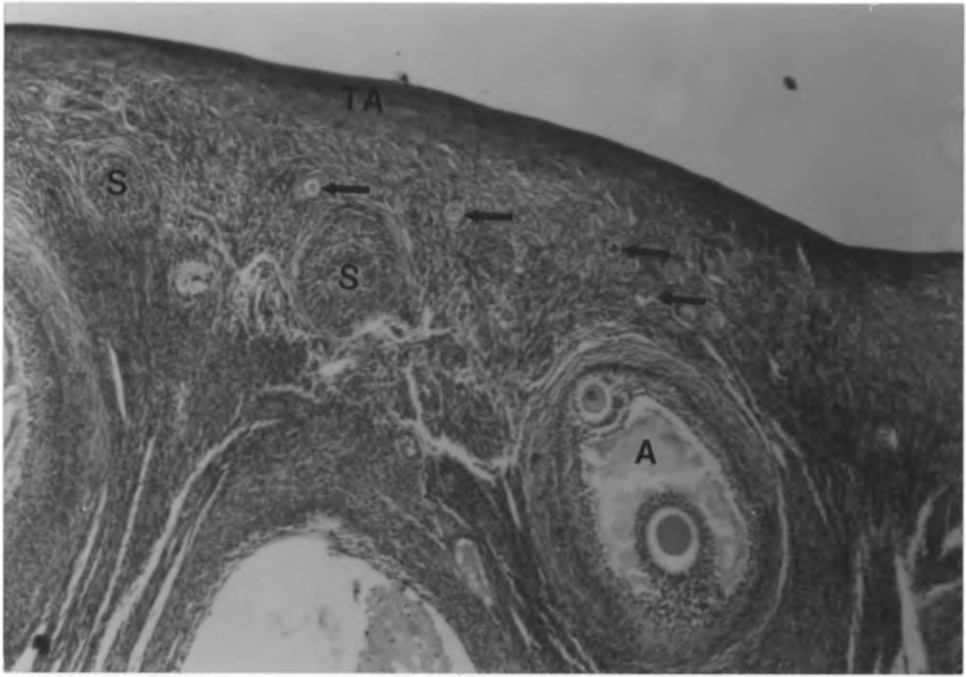


Figure 9 Typical histology of ovaries from pig ZP group as represented by 2044 (day 273 of ovariectomy)

Magnification X 40; Filter LBD

Legend

A	: Antral (tertiary) follicle
S	: Secondary follicle
TA	: Tunica albuginea
Arrow	: Primordial, primary or early secondary follicle

Note that due to the low magnification, differentiation between primordial, primary and early secondary follicles is not possible.

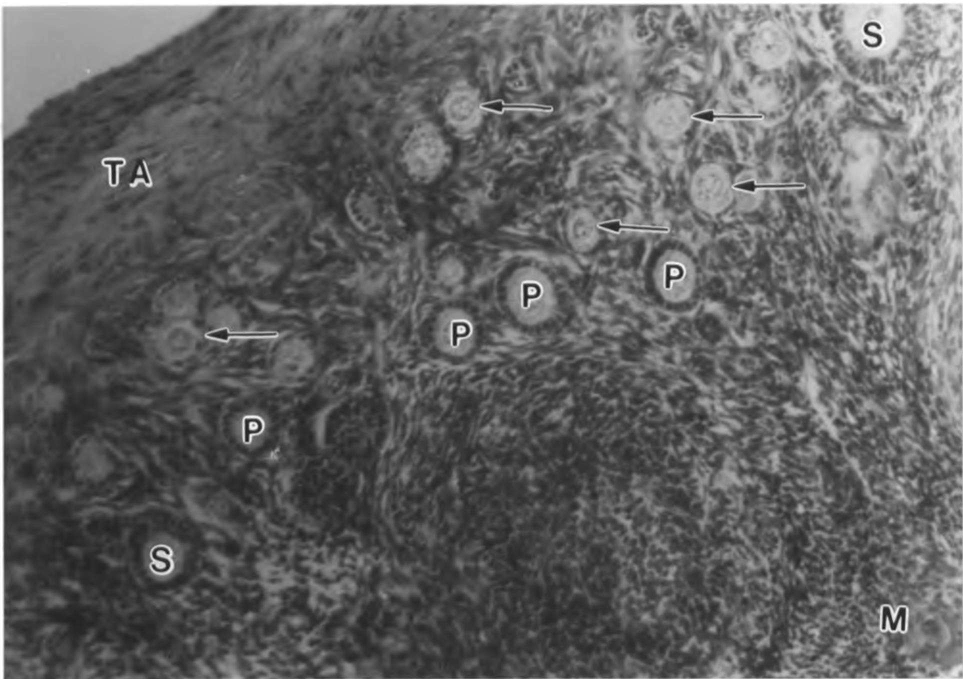


Figure 10 Typical histology of ovaries from rabbit ZP group as represented by 1997 (day 275 of ovariectomy)

Magnification X 100; Filter LBD

Legend

M	: Ovarian medulla
P	: Primary follicle
S	: Secondary follicle
TA	: Tunica albuginea
Arrow	: Primordial follicle

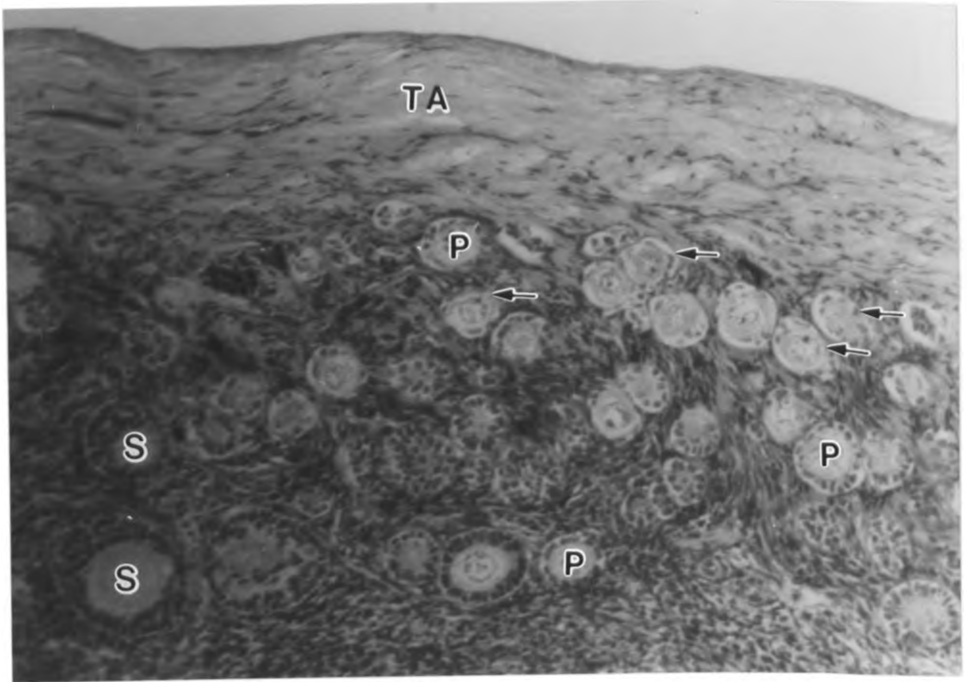


Figure 11 Typical histology of ovaries from control group as represented by 2033 (day 273 of ovariectomy)

Magnification X 100; Filter LBD

Legend

P	: Primary follicle
S	: Secondary follicle
TA	: Tunica albuginea
Arrow	: Primordial follicle

Table 6 Summary of histological analysis of ovarian sections

Animal #	Treatment	Day of O*	Primordial follicles	Primary follicles	Secondary follicles	Tertiary follicles
1994	PZP	50	+N	+N	+N	+N
2032	PZP	61	+N	+N	+N	+N
2016	Control	121	+N	+N	+N	+N
2020	PZP	121	+N	+N	+N	+N
1991	RZP	183	+N	+N	+N	+N
2001‡	RZP	183	+N	+N	+N	+N
2033	Control	273	+N	+N	+N	+N
2044	PZP	273	+N	+N	+N	+N
1992‡	RZP	275	+N	+N	+N	+N
1997	RZP	275	+N	+N	+N	+N
2025	Control	365	+N	+N	+N	+N
2020	PZP	365**	+N	+N	+N	+N

Key

#	: Number
*	: Day of ovariectomy is dated from the day of primary immunization with this day taken as day 0
**	: Terminal point for this animal whose second ovary was removed on day 365
+N	: Normal follicles present
‡	: Received only one boost instead of the required two boosts of immunogen
RZP	: Immunized with rabbit zona pellucida
PZP	: Immunized with pig zona pellucida

Despite the gross morphological findings, the histology from the two experimental groups was comparable to that of the control group. Clearly, normal follicles in all stages of development were seen in each animal of each group. This shows that immunization with either native rabbit ZP or native pig ZP, using TitreMax® as adjuvant, did not alter normal ovarian histology in the female baboons. These histological results were ascertained by the cycle chart data (Table 7 below and Appendices 7-17).

3.6 Ovarian cyclicity as determined by sex skin scoring

All the animals of the study continued to have normal ovarian cyclicity as ascertained by sex skin scores. There was no discernible difference between the pre- and post-immunization cycles of the ZP-immunized groups (pig ZP and rabbit ZP). The same was true when the post-immunization cycles of the two groups were compared with those of the control group. Table 7 below gives a summary of the cycle lengths of the three groups of animals for the entire length of study. Cycle charts detailing the same (for three months pre- and five months post-immunization) are contained in Appendices 7-17.

Table 7 Summary of effects of immunization of female baboons with zona pellucida antigens on cycle lengths

Animal number	Average cycle length BI (D)	Average cycle length PI (D)
2016*	34.5	34.0
2025*	35.6	35.9
2033*	39.5	39.0
1991†	40.4	41.2
1992†	36.3	36.5
1997†	33.5	34.0
2001†	37.6	36.9
1994‡	38.0	39.1
2020‡	40.5	41.0
2032‡	46.4	45.9
2044‡	35.9	36.4

Key

BI : Before immunization

D : Days

PI : Post immunization

* : Control animals

† : Immunized with rabbit zona pellucida

‡ : Immunized with pig zona pellucida

Note that animal numbers 1992 and 2001 received only one boost instead of the required two boosts of immunogen.

These results of ovarian cyclicity indicate that all the animals continued to cycle normally for the duration of the study. However, due to the shorter period of evaluation of the rabbit ZP group in comparison to the pig ZP and control groups, comparable long-term effects of immunization with native rabbit ZP in TitreMax® adjuvant on ovarian cyclicity could not be ascertained. However, for the period of evaluation for the pig ZP group (12 months) and the rabbit ZP group (9 months) ovarian cyclicity as evidenced by sex skin scoring was normal. It is also expected that by nine months, the antibody titres, if any, would be negligible and thus the effects on ovarian cyclicity, if any, would be absent.

CHAPTER 4. DISCUSSION

4.1 ELISA results

The ELISA results demonstrated that both native heat solubilized pig and rabbit ZP are immunogenic in female baboons using TitreMax® adjuvant as shown by the induction of antibodies against the immunogens. Due to lack of adequate native antigen to perform ELISA experiments, sera from each animal's bleeds for the first 5 months post-immunization were pooled and this used for a one-point determination of antibody titres. The sera revealed that the rabbit ZP group developed higher antibody titres, as evaluated by solid phase ELISA, than the pig ZP group indicating that the rabbit ZP is a better immunogen at least in the baboon, using TitreMax® as adjuvant. Although direct comparison to absolute titres in other studies is not practical due to the tremendous variation in ZP immunogens and dosages, injection regimes and titration assays used, the antibody titres obtained in the two groups were within the range of those obtained in similar animal studies. However, considerable variation exists in antibody titres among the various studies and the probable reasons for this will be discussed using the following examples.

The study by Wood *et al.* (1981) showed that the majority of rabbits heteroimmunized with variously treated forms of native pig ZP developed elevated serum titres after the first booster (3-4 weeks after the primary immunization) and that these levels remained remarkably consistent throughout the remainder of the study which took slightly over 210 days. The immunization regimen consisted of 300 µg of ZP antigen for the primary immunization followed by 150 µg for the first boost at

3-4 weeks and 75 μg for the second and third boosts at weeks 9-11 and 24-26 respectively. The study of Sacco *et al.* (1987) also showed that after immunization of squirrel monkeys (*Saimiri sciureus*) with 200 μg each of a purified preparation of the 55,000 macromolecule (ZP3) from pig ZP, antibody titres remained high even 693 days after the initial immunization. On the other hand, other studies have reported low titres of antibodies as evaluated by ELISA. For example, Jones *et al.* (1992) showed that immunization of rabbits with deglycosylated ZP macromolecules of pigs resulted in lower titres of anti-ZP antibodies as compared with the fully glycosylated forms. They thus concluded that the carbohydrate moieties are the ones involved in production of higher antibody titres.

To support this, the study of Schwoebel *et al.* (1992) showed that immunization of Cynomolgus monkeys with recombinant rabbit ZP proteins that are not glycosylated resulted in undetectable antibody titres, on ELISA assay, after primary and two booster injections. However, increasing the immunogenicity of these proteins by conjugating them to either protein A or Keyhole Limpet Hemocyanin resulted in measurable antibody titres. However, even after conjugation, the recombinant 75b still elicited very low or no antibody titres. These investigators thus concluded that this portion of the 75-kDa protein is not immunogenic by itself. The study of VandeVoort *et al.* (1995) which was a continuation of that of Schwoebel *et al.* (1992) verified the low immunogenicity of recombinant proteins shown by the latter investigators. VandeVoort *et al.* (1995) also had to conjugate the recombinant proteins to protein A and in addition, immunize the monkeys monthly for another 12

months to maintain the low antibody titres induced in the study of Schwoebel *et al.* (1992).

These differences in antibody titres among the various studies could be attributed to the following factors. The immunogenicity and antigenicity of the ZP is very complex and depends on the species of ZP and animal used for immunization as pointed in the review by Dunbar *et al.* (1986). The different studies have used various species of ZP for immunization (for example pig ZP- Wood *et al.*, 1981, Mahi-Brown *et al.*, 1982, 1985, Sacco *et al.*, 1987, Jones *et al.*, 1992; rabbit ZP- Wood *et al.*, 1981; rat ZP- Mahi-Brown *et al.*, 1992) and several species of animals for immunization (for example squirrel monkey- Sacco *et al.*, 1983a, 1987, 1989; rabbit- Wood *et al.*, 1981, Jones *et al.*, 1992; dog- Mahi-Brown *et al.*, 1982, 1985). In the present study where rabbit and pig ZP immunization were compared in the female baboon, rabbit ZP was found to elicit higher antibody titres thus confirming that the species of ZP used also contributes to the type of immunogenic response.

In addition, it has further been shown that the nature of the ZP antigen also causes variation in the antibody titres. For instance, Sacco *et al.* (1986b) and Henderson *et al.* (1987b) showed that the carbohydrate chains of the ZP glycoproteins influence the antigenic and immunogenic properties with deglycosylated ZP proteins being less immunogenic (lower titre antibodies) than the native glycosylated ZP proteins. Similar results were also observed by Jones *et al.* (1992), Schwoebel *et al.* (1992) and VandeVoort *et al.* (1995). In their review, Dunbar *et al.* (1994) stated that the variation in these immune responses most likely reflects differences in the ZP proteins at the amino acid level and at the level of

post-translational modification. This low immunogenicity of various forms of ZP antigens can however be improved. In a classical example, Prasad *et al.* (1995), expressed the cDNA for the rabbit 55-kDa ZP protein in insect cells (Sf9) with recombinant baculovirus to obtain the nonfusion glycosylated recombinant protein (BV-55). They did this since nonglycosylated, unconjugated recombinant ZP proteins are poor immunogens (Schwoebel *et al.*, 1992; VandeVoort *et al.*, 1995). Immunization of female rabbits with this protein resulted in appreciable antibody titres in contrast to immunization with rec55 protein produced in bacteria hence showing the importance of glycosylation. In addition, alloimmunization with native rabbit ZP antigens (Wood *et al.*, 1981) does not result in detectable antibody titres. The glycosylation of BV-55 protein therefore, differs sufficiently from glycosylation of the 'self' ZP molecule to elicit antibody production. BV-55 protein was also shown to be as immunogenic as the native 55-kDa protein (when heteroimmunized) as evidenced by the generation of a high titre of antibodies to native protein in guinea pigs immunized with BV-55 protein. The study of Prasad *et al.* (1995) therefore, shows the possibility of using 'self' antigens for immunization. In addition, the present study, as do all ZP studies to date, also shows that individual variation in antibody titres exists even amongst a species in response to immunization by ZP antigens. It is thus of prime importance to generate antigens that will result in efficacious antibody titres in all individuals.

As indicated in the review of Dunbar *et al.* (1986), the immunogenicity and antigenicity of the ZP also depends on the method used to solubilize and purify the proteins. The various studies have used different methods to solubilize and purify the

ZP antigens that they have used for immunization. For example, Schwoebel *et al.* (1992) solubilized the ZP glycoproteins by heating for 1 hour at 68 °C in a diluted sodium carbonate solution, pH 9.5 while Skinner *et al.* (1984) used a 0.4 mM sodium carbonate buffer, pH 9.0 at 60 °C for 1 hour. On the other hand, Sacco *et al.* (1987) isolated ZP3 by heat-solubilizing zonae pellucidae at 73 °C for 20 minutes followed by treatment with 3% sodium dodecyl sulfate and heat (60 °C, 20 minutes) under nonreducing conditions. The ZP3 glycoprotein was isolated by column chromatography using first Sephacryl S-400 and next, hydroxylapatite resins equilibrated with buffer containing 0.1% SDS. Purity of the final ZP3 preparation was verified by 2D-PAGE. Jones *et al.* (1992) heat solubilized isolated zonae pellucidae at 73 °C for 20 minutes and prepared ZP3 in a method very similar to that of Sacco *et al.* (1987) described above. The only difference was that heat solubilization was performed for 10 minutes more. Dunbar *et al.* (1989) purified two pig ZP glycoproteins by either preparative isoelectric focusing or preparative SDS-PAGE. Most of the studies used 2D-PAGE to monitor the purity of the preparations as this procedure has been shown to be essential for the resolution of ZP glycoproteins. In addition to differences in solubilization and purification methods, the differing times and buffers used for the heat solubilization method could also account for the observed variations in the immunogenicity of the molecules amongst the various studies.

It also should be noted that though heat solubilization represents one of the mildest and least disruptive methods for obtaining ZP components in solution, the antigenic integrity of some ZP components is however lost using this method (Sacco

et al., 1983b). In this study, Sacco *et al.* (1983b) produced serum against intact ZP which is the most likely to contain antibodies developed against ZP antigens in their native and unaltered configuration within the intact ZP. The antiserum recognized six antigens in heat solubilized ZP preparations while the intact ZP had eight antigenic determinants.

The immunization regimes used in these studies are also different. For example, the number and time interval of the boosts after the primary immunizations are not the same (see above studies). In addition, differing routes of administration of the ZP antigens and quantity of injection solution are used across these studies. For example, Schwoebel *et al.* (1992) and VandeVoort *et al.* (1995) used subcutaneous mode of administration while Sacco *et al.* (1987, 1989) used intradermal. In the current study, the subcutaneous route was used. It is well known that the mode of administration of an antigen has effects on the immunogenic response. With regard to the amount of solution, the study of Sacco *et al.* (1989) injected 1 ml at each immunization; Schwoebel *et al.* (1992), 1 ml also; this study 200 μl; while others did not report the amount of solution injected (Sacco *et al.*, 1987).

In addition, differing methods of protein quantification were used among different studies making accurate comparison difficult. For example, Schwoebel *et al.* (1992) used the method of Lowry *et al.* (1951) to determine protein content of native ZP glycoproteins while most other studies did not report the methods of protein quantification used (Skinner *et al.*, 1984; Rhim *et al.*, 1992; Bagavant *et al.*, 1994). The quantities of ZP antigens used for immunization across the studies have also varied and thus contributed to the observed differences in antibody titres. The

study of Sacco *et al.* (1989) clearly summarizes this. These investigators evaluated the effect of immunization with varying dosages of porcine zona pellucida Mr=55,000 (ZP3) glycoprotein on antibody response in female squirrel monkeys. They used dosages of 50 μg, 25 μg and 5 μg. One half of the total dosage was given over a series of three immunizations spaced one week apart and the rest was given in a single booster injection after peak titres were obtained. They obtained dose-dependent titration profiles in contrast to earlier findings in the squirrel monkey system (Sacco *et al.*, 1986b) where no significant differences were observed when antibody response to the ZP3 dosage ranges of 2.0, 1.0 and 0.5 mg and 400, 200 and 100 μg were compared. Thus, these data collectively indicate that at least for the squirrel monkey system, when using ZP3 as immunogen with the indicated immunization regime, a maximal immune response is induced throughout the dosage range of 2.0 mg to 100 μg, and a dose-dependent response takes place only below the 100 μg level. Thus, similar studies are needed in various animal models to determine the minimum amount of ZP antigen needed to obtain a maximal antibody response.

The type of adjuvant used is also critical in determining the levels of antibody titres. As indicated by Schwoebel *et al.* (1992), it is likely that the properties of the adjuvant are a major factor in the maintenance of antibody levels in addition to the immunogenicity of the protein used for vaccination. Most ZP studies to date have used either Freund's adjuvant, aluminium salts or a muramyl dipeptide analog (MDP) as adjuvant. Thus, the study of Sacco *et al.* (1989) which compared the effects of these adjuvants summarizes the situation effectively. Female squirrel monkeys received 200 μg of pig ZP3 in either of the three adjuvants. MDP was found to be as

effective as Freund's in eliciting high titres of antibodies. On the other hand, aluminium hydroxide was not an effective adjuvant in this system although other studies using this adjuvant (Gulyas *et al*., 1983; Mahi-Brown *et al*., 1985) with pig ZP immunogens obtained at least a moderate titre response.

The findings of Sacco *et al*. (1989) are not isolated. Van Nest *et al*. (1992) reported that although alum has an excellent safety record and that it has proved useful for some vaccines, its relatively weak immunostimulatory activity makes it a suboptimal adjuvant for recombinant subunit vaccines, in which direction ZP-based studies are geared. This is of considerable concern since alum is the only adjuvant approved for human vaccines in many countries. Powerful adjuvants classically used in research such as complete Freund's adjuvant (CFA) generate side effects far too severe to be used in human-based vaccine applications. Clearly, an adjuvant with immunostimulatory activities similar to CFA but with low side effects similar to alum would be the ideal choice for use with human subunit vaccines. This then highlights the need for increased studies in adjuvant formulations for ZP-based vaccines.

Indeed, Van Nest *et al*. (1992) found that low-oil adjuvant formulations containing squalene, surfactants and the muramyl peptide, MTP-PE are effective with a wide variety of recombinant and natural subunit antigens. These formulations generated antibody titres much higher than alum or incomplete Freund's adjuvant (IFA). Unstable, large-droplet size emulsions were effective in small species such as rodents and rabbits but were ineffective in stimulating antibody responses in large animal species such as goats, baboons, chimpanzees and humans. The large animal species required stable adjuvant emulsions of small droplet size (less than 1 μm) for

effective stimulation of antibody responses. This may explain the results observed with the adjuvant (TitreMax®) used in the current study. The titres obtained in this study were appreciable and there were no observable side effects. This adjuvant is formulated with squalene (a metabolizable oil) and the active ingredient, CRL89-41, is bonded to silica particles that act as stabilizers for the water-in-oil emulsion. Though this adjuvant was not compared with another in the same study, comparison with results of other studies show that it should be further evaluated for use in ZP-based vaccines for human application.

Apart from showing induction of antibodies against the respective immunogen, the ELISA results also showed that two of the four baboon anti-native pig ZP sera had low titres against native rabbit ZP. Similarly, two of the four baboon anti-native rabbit ZP sera had low titres against native pig ZP. Indeed, Dunbar *et al.* (1994) obtained comparable results in a similar experiment. In their study, they showed that antibodies raised in the guinea pig against native pig ZP recognized native ZP proteins in both the rabbit and pig whereas antibodies raised against rabbit ZP recognized native rabbit ZP proteins and only some pig ZP proteins. These results confirm, therefore, the cross-reactivity that exists between the two species of ZP that were under test in this study. However, it is interesting to note that not all the animals' sera recognized the cross-reacting immunogen on ELISA assay. Various reasons can be put across for this. Firstly, the two animals of the rabbit ZP group (numbers 1991 and 1997) that recognized pig ZP had the highest titres of antibodies and so it is probable that the other animals' antibody titres were too low to give positive results. The latter two animals (numbers 1992 and 2001) received only one

boost of immunogen instead of the required two boosts. However, the same can not be said for the pig ZP group. In this group, the sera of animal number 1994 did not recognize rabbit ZP although it had comparable antibody titres to animal number 2020 whose sera recognized rabbit ZP. The situation is further complicated by the fact that animal number 2032 (pig ZP group) which was the other animal whose sera recognized rabbit ZP had low antibody titres (fourfold less than animal number 2020). Thus, for the rabbit ZP group, there is an apparent correlation between the level of antibody titres and recognition of pig ZP. The same however, can not be said for the pig ZP group and recognition of rabbit ZP. It can therefore be concluded that individual variation exists in response to immunization by ZP antigens. Other conclusions- masking of shared epitopes and nature of the immune response- as discussed below for immunoblot results may also hold true. The occurrence of cross-reactivity between rabbit and pig ZP as determined by ELISA, was also verified by immunoblot analysis as discussed below.

4.2 Immunoblot results

In immunoblot analysis, the antibodies from the rabbit ZP group recognized both native rabbit and pig ZP in addition to bacterially produced recombinant protein (BPRP) encoding the 55-kDa protein and both portions of the 75-kDa protein of the rabbit ZP antigen. On the other hand, antibodies from the pig ZP group all recognized native pig and rabbit ZP in addition to BPRP protein encoding the 75a portion of the 75-kDa protein of the rabbit ZP antigen. The recognition of corresponding native protein shows that immunization with ZP antigens produces antibodies that recognize the immunogen. To date, all ZP studies apart from those where immunogenicity of the

antigens were low (Schwoebel *et al*., 1992; VandeVoort *et al*., 1995) confirm this. It is of great interest that the antibody populations of the pig ZP group were very specific in their recognition in immunoblot analysis of the recombinant rabbit ZP antigens. They only recognized the 75a portion of the 75-kDa protein. On the other hand, all the antibody populations from the rabbit ZP group recognized all the recombinant rabbit ZP proteins under test; 55-kDa and the 75a and 75b portions of the 75-kDa protein. Several inferences can be made from these results.

Most portions of the ZP molecule are antigenic (can bind antibodies) since all the antibody populations (at least from the rabbit ZP group) recognized all the recombinant rabbit proteins under test in this study. However, the native ZP proteins under test on immunoblot analysis were whole proteins and not individual ones and from this it is therefore impossible to know which portions of the ZP molecule were recognized by the antibody populations. The use of individual recombinant proteins on immunoblot analysis, on the other hand, allowed the evaluation of the antigenicity of individual rabbit ZP proteins. Since rabbit ZP is also composed of another protein, the 45-kDa (Dunbar *et al*., 1994) whose recombinant was not available for test it can only be concluded that most portions of the rabbit ZP are antigenic. Schwoebel *et al*. (1992) showed that though the recombinant 75b was not immunogenic (did not induce antibody formation) by itself, it was antigenic in that antibodies against total native rabbit ZP protein recognized it.

The immunoblot results also show that recombinant rabbit ZP proteins contain epitopes found in the native rabbit ZP. Though this is expected, it is important to confirm the same. This is because the recombinants are produced in bacteria as

opposed to eukaryotic cells and are thus linear and sequential antigens. The mature native ZP of eukaryotic cells is however, composed of conformational as well as sequential antigenic determinants (Dunbar and Raynor, 1980; Sacco *et al.*, 1983b; Drell and Dunbar, 1984). This study thus shows that the differences between the two types of proteins did not inhibit binding of recombinant proteins to antibodies against native ZP proteins (antigenicity). Similar studies by other researchers confirm this. Lee *et al.* (1993) showed that the recombinant 75a protein contained epitopes found in the native rabbit ZP glycoprotein. VandeVoort *et al.* (1995) also showed that immunization with pEX-expressed recombinant 75a, 75b and 55 rabbit ZP antigens resulted in antibody populations that recognized native rabbit ZP.

Another inference from the immunoblot results is that the recombinant 75a protein of rabbit ZP contains epitopes found in the native pig ZP. ELISA and immunoblot results showed that cross-reactivity exists between native rabbit and pig ZP. From these results using recombinant proteins it can be concluded therefore, that the level of cross-reactivity between native pig and rabbit ZP is at the 75a portion. This factor of cross-reactivity is of great importance because the possibility exists that only one form of ZP antigen may eventually be used in the production of a contraceptive vaccine for the majority of female species. It is however surprising that the antibodies from the pig ZP group did not recognize rec55 protein since Lee *et al.* (1993) showed similarity in deduced amino acid sequences of the rec55 and rec75 cDNA clones. In addition, Schwoebel *et al.* (1992) showed that antibodies against rec75a peptide recognized both the native 75-kDa and 55-kDa rabbit ZP proteins while antibodies against native 55-kDa protein recognized both native 75-kDa and

55-kDa proteins. The possible reasons for this anomaly are discussed in the four points below.

Firstly, antibodies may not have been produced against the shared epitopes. These shared epitopes could have been masked in the immunogen and so probably no antibodies were produced that recognized rec55. Secondly, it is possible that rec75a is expressed in a greater ratio of recombinant protein to bacterial protein than the rec55, and therefore, the shared epitopes may be more concentrated in the rec75a. Thirdly, it is also important to note that the immunoblotting procedure could only test the humoral response to immunization using ZP antigens and not the cellular response. Thus it could also be possible that only a cellular response was generated against the rec55 and so on immunoblot analysis using the produced antibodies, this recombinant protein could not be picked up by the antibodies. The study of Mahi-Brown *et al.* (1992) confirm this in that they showed that both a humoral and a cellular response result from immunization with ZP antigens. Finally, several studies (Sacco *et al.*, 1986b; Henderson *et al.*, 1987b; Jones *et al.*, 1992) have shown that the carbohydrate moiety of the ZP is of greater importance than the protein moiety in the immunogenicity of the ZP antigen.

In some of these studies where deglycosylated ZP antigens were used for immunization the resultant levels of antibodies were quite low. Therefore, it could be probable that the antibody populations produced as a result of immunization with the native ZP antigens in this study were mainly directed at the carbohydrate moieties rather than the protein ones and that levels directed at the protein moieties were probably very low. The recombinant proteins, not being glycosylated, could therefore

not be easily picked on immunoblot analysis. If this is true for all the constituent proteins of the ZP antigen, then it would be expected that none of the recombinant proteins would be recognized by any of the antibody populations which was not the case. Thus the probable reasons for this observation could be either that the recombinant 55 rabbit ZP antigen that was not picked by immunoblot analysis using anti-total native pig ZP antibodies did not have any or had a low level of humoral response produced to its corresponding native counterpart.

To ascertain where the level of cross-reactivity between native rabbit and pig ZP exists using the population of antibodies in this study, it would be necessary to do the following. The native ZP antigens used for immunoblotting should be separated by 2D SDS-PAGE into their individual glycoproteins and these then probed with the various populations of antibodies. The importance of this is further highlighted by the review of Dunbar *et al.* (1994) which states that pig ZP3α and rabbit 55-kDa ZP protein share 66% homology. It is expected that the antibody populations recognizing a particular individual native glycoprotein should also recognize the corresponding recombinant rabbit ZP protein.

The fact that all the animals in each experimental group gave the same results on immunoblot analysis points to the fact that the response to immunization with ZP antigens is very specific. In addition, the differences in the results between the two groups confirms, as had been stated earlier, that the immunogenic response depends not only on the animal species being immunized but also on the species of ZP used for immunization. This also stresses the importance of testing multiple species of ZP in

multiple species of animals in order to produce as complete data as possible on the immunogenic response to immunization with ZP antigens.

From the immunoblot results, it is clear that the use of whole native ZP as opposed to recombinant fragments to immunize in preliminary studies, allows the analysis of the immunogenicity of the whole molecule and not just portions of it as the testing of individual recombinant proteins does. This is because the antibody populations directed against the whole native protein can be used to determine, using individual constituent proteins of native ZP produced by recombinant technology, the epitopes of the native ZP that are immunogenic in any given species. From this point then, the recombinant proteins identified can be used in further studies to assay their effects in immunized animals. This path could provide one way of minimizing the expenses, both in monetary and temporal terms, involved in experimentation. It is important to note that this study is also the first to evaluate the antigenicity of antibodies produced in response to immunization with native ZP antigens to recombinant rabbit ZP proteins. Consequently, it is therefore apparent that more studies are needed in this area in order to draw more conclusive evidence.

To be used as an immunocontraceptive agent, the ZP immunogen should produce antibodies against the ZP of the animal under test. To evaluate if this was the case in this study, immunohistochemistry was employed.

4.3 Immunohistochemical results

Normal baboon ovarian sections were probed with immune sera from all the immunized animals. The results were a positive staining of the baboon ZP for the

ZP-immunized groups and not for the control group. In addition, the oocyte cytoplasm and surrounding layer of granulosa cells were also stained. This shows that autoantibodies were produced in response to immunization with either pig or rabbit ZP. It should be noted that the staining pattern depended on the stage of development of the follicle and thus of the oocyte and its ZP irrespective of the type of immune sera used for probing. For example, Figure 7 (baboon ovarian section probed with baboon anti-pig ZP sera), shows a relatively immature oocyte whose ZP was still undergoing development. In addition, numerous granulosa cells and the oocyte cytoplasm are deeply stained. To corroborate this, Figure 5 (baboon ovarian section probed with rabbit anti-pig ZP sera), also shows lack of clear delineation of the ZP of the immature oocytes. In addition, their oocyte cytoplasm is appreciably stained while the surrounding granulosa cells are very deeply stained. On the other hand, Figure 8 (baboon ovarian section probed with baboon anti-rabbit ZP), shows an almost mature oocyte whose ZP is clearly defined. In addition, a lesser number of granulosa cells have stained and the oocyte cytoplasm is hardly stained.

These results show therefore, that immunohistochemistry is an important tool in the study of ovarian follicular development. Other studies have also underscored the importance of immunohistochemistry as an elucidatory tool in ZP experiments. For example, Dunbar *et al.* (1994) probed growing rabbit follicles with sheep polyclonal antibody raised against total rabbit ZP. The inner layer of granulosa cells in addition to the ZP of secondary follicles were stained. In an even more elaborate experiment, Hinsch, K-D *et al.* (1994) generated antisera against synthetic peptides corresponding to conserved or mouse-specific ZP3 epitopes. Using antisera against a

specific conserved epitope, AS ZP3-5, positive reaction was obtained with ZP of all mammalian oocyte sections tested including mouse, rat, hamster, pig, cow and human. Both the ZP and oocyte were stained except for rat ooplasm and cumulus cells of human and bovine. In addition, none of the antisera reacted with other cells of the ovary or of any other tissues tested, including the oviduct, uterus, kidney, adrenal, liver, heart, skeletal muscle, brain, spleen, lung, stomach, small intestine and gut. Koyama *et al.* (1985) showed that immunization of mice with pig ZP resulted in antibodies that caused immunofluorescence of pig ZP. Henderson *et al.* (1987a) showed that polyclonal antibodies to a 32-kDa deglycosylated peptide from pig ZP will result in immunofluorescence of human oocytes.

As in immunoblot analysis, the immunohistochemical results also showed that sera from the rabbit ZP group displayed stronger signals than the pig ZP group. This could either mean that the antibodies induced by the native rabbit ZP were more proficient at recognizing baboon ZP than those induced by native pig ZP and/or native rabbit ZP was a better immunogen than native pig ZP and thus elicited higher titres of antibodies (as correlated by the ELISA data) hence a more positive signal. These two hypotheses are supported by the fact that the immune sera of the two rabbit ZP animals (numbers 1992 and 2001) that received only one boost instead of the required two boosts, gave a positive immunohistochemical signal. On the other hand, comparable immune sera from the pig ZP group (sera after the first boost) resulted in a negative immunohistochemical signal. Since the study reported in this thesis is the first to comparatively probe normal baboon ovarian sections with baboon anti-native

pig ZP and baboon anti-native rabbit ZP sera, it is evident that more studies are needed in this area in order to draw more conclusive evidence.

However, these immunohistochemical studies show that the immune tolerance to the native ZP antigen can be broken using such a regimen of immunization with a cross-reacting but 'foreign' species of ZP. They also show the possibility of dissecting the antigenic and immunogenic components of the native ZP. In addition, immunohistochemical studies of the formation of the ZP is an excellent model system to study the early stages of follicular development and cell differentiation. The study of Wolgemuth *et al.* (1984) was the first to use well-characterized antibodies to electrophoretically purified ZP proteins to analyze the appearance and formation of the extracellular ZP glycoprotein matrix. Since then, such studies have become more detailed. For example, Dunbar *et al.* (1994) used a monoclonal antibody specific for a carbohydrate-associated antigen (PSI) of the pig in such studies. Indeed, it was the tool of immunohistochemistry that Timmons *et al.* (1990) used to show that PSI is located in lipophilic granules of the oocytes and within discrete layers of the developing ZP from its earliest appearance in the follicle. In addition, tissue fixation techniques can also be evaluated and optimized using this experimental tool. For example, Skinner and Dunbar (1992) showed that fixation with formaldehyde stabilizes the antigenic determinant, PSI so that it is associated with distinct granules in the oocyte cytoplasm. For ZP-based studies, the prime importance of immunohistochemistry is the identification of the specific cell types that synthesize the ZP and the understanding of the assembly of the ZP during follicular development. It should be possible to identify distinct antigenic epitopes that are associated with the

ZP matrix following the differentiation of the steroid producing granulosa cells in a bid to bypass ovarian pathology. Such studies will be crucial in the eventual realization of the ZP as a potential immunocontraceptive for women.

These immunohistochemical results further show the extensive cross-reactivity among various species of ZP. In this study, cross-reactivity was shown to exist between all species of ZP used; rabbit ZP and pig ZP (ELISA and immunoblot analysis of recombinant rabbit ZP antigens), rabbit ZP and baboon ZP (immunohistochemical analysis) and baboon ZP and pig ZP (immunohistochemical analysis). Many studies have shown cross-reactivity between a great assortment of ZP species including human and pig (Koyama *et al.*, 1985; Henderson *et al.*, 1987; Hasegawa *et al.*, 1988). This indicates that the ZP is a partially conserved molecule and as discussed earlier, a possible advantage exists in that only one form of ZP antigen may eventually be used to produce a contraceptive vaccine for females of all species of mammals.

So far, the study has shown that immunization of female baboons with either pig or rabbit ZP results in the production of antibodies that not only recognize the immunogens and recombinant rabbit ZP antigens but also native baboon ZP. The induction of autoantibodies is critical for a potential immunocontraceptive. However, what are the effects of these antibodies on ovarian morphology and function?

4.4 Effects of induced antibodies on ovarian morphology and function

Though there was an apparent correlation between cystic ovaries and ZP-immunized animals, these results should be interpreted with caution as some investigators have routinely found cystic ovaries in normal baboon ovaries (Dunbar,

B. S., personal communications, 1996). In addition, only one animal (number 2020) of the pig ZP-immunized group had cystic ovaries. This animal had both ovaries removed. Thus, the cysts could be purely coincidental. In addition, if the cysts were due to the immunogens, it would be expected that the two rabbit ZP-immunized animals (numbers 1991 and 1997) that received two boosts to be more prone to cysts than those that received only one boost. This, however, was not the case as cysts were found in one animal of each group (boosted once- number 1992 or twice- number 1997). However, the number of animals- one for each time period- is too small to give conclusive evidence on the gross morphological findings. Though the literature is scant with respect to ovarian cysts in baboons some researchers have rarely (Hendrickx, A. G., personal communications, 1996) or routinely (Dunbar, B. S., personal communications, 1996) encountered cystic ovaries in normal baboon ovaries. In view of all the above, these results should therefore be interpreted with caution. Further work therefore, needs to be done in this area. It is evident that in future studies, laparascopy would be an invaluable tool for monitoring ovarian morphology *in vivo*, both before and during experimentation.

To elucidate the significance of these gross morphological findings on ovarian histology, sections of the ovaries were subjected to analysis of follicle status. It was evident that the ovarian histology of the two experimental groups was comparable in all aspects to that of the control group. This was further verified by the cycle data in which all the animals continued to cycle normally.

These results are in contrast with the study of VandeVoort *et al.* (1995) in which immunization of Cynomolgus monkeys with the recombinant rabbit 75-kDa

ZP protein resulted in the production of antibodies that interfered with ovarian follicular development and ovarian cyclicity while immunization with the recombinant rabbit 55-kDa ZP protein resulted in antibodies that did not affect ovarian follicular development or subsequent ovarian hormonal cyclicity. In the current study, both the rabbit ZP group whose antibodies recognized all the recombinants in addition to the rec55 protein and the pig ZP group whose antibodies recognized the rec75a protein (the only recombinant recognized) did not show any ovarian pathology. The ovarian function results are also in contrast to the similar study of Dunbar *et al.* (1989) where 2 out of the 5 experimental baboons became amenorrheic by 8 months following immunization with denatured and deglycosylated porcine ZP glycoproteins in a synthetic peptide adjuvant CGP-11,637. Other studies using various ZP immunogens and various species of animals for immunization have shown either an interference (Skinner *et al.*, 1984; Jones *et al.*, 1992; Paterson *et al.*, 1992; Rhim *et al.*, 1992) or non-interference (Sacco *et al.*, 1987; Jones *et al.*, 1992; Bagavant *et al.*, 1994; Tung *et al.*, 1996) with normal ovarian function. These studies then continue to point to the fact that not only does the nature of the ZP protein used in immunization affect the outcome of ovarian function but that also the adjuvant may have an effect.

Indeed, some studies have shown that the properties of the adjuvant are a major factor in the resultant effects of ZP immunization on ovarian function. For example, Upadhyay *et al.* (1989) investigated the role of adjuvants in inhibitory influence of immunization with porcine zona pellucida antigen (ZP3) on ovarian folliculogenesis in bonnet monkeys and compared the effect of two adjuvants; complete Freund's adjuvant (CFA) or sodium phthalylated lipopolysaccharide

(SPLPS). They found that although no morphological damage to ovarian components was observed in animals immunized with SPLPS as adjuvant, immunization using CFA resulted in profound ovarian follicular atrophy, sparing only the primordial follicles. On the other hand, Bagavant *et al.* (1994) demonstrated that ZP3 immunization of bonnet monkeys (*Macaca radiata*) using either (SPLPS) or alum mixed with muramyl dipeptide (MDP) did not result in any obvious ovarian changes. These two studies are thus in agreement regarding SPLPS in that it does not result in ovarian damage. It is therefore probable that the lack of overt histopathology in the present study could also be contributed by the type of adjuvant used. It is therefore imperative that investigations for suitable adjuvants for ZP-based contraceptive vaccines be continued.

4.5 Conclusions

In summary, the overall results of this study show that immunization of female baboons with either native pig or rabbit ZP in TitreMax® adjuvant results in antibodies that recognize the respective immunogens and the cross-reacting native ZP. In addition, the antibody populations recognize the ZP of the animal under test (baboon) plus recombinant rabbit ZP antigens. These antibody populations do not result in ovarian pathology.

Recommendations for further areas of work arising from the results of this study include the following.

1. Characterization of the immune response elicited in this study into humoral and cellular arms.

2. Two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D SDS-PAGE) separation of native rabbit and pig ZP protein and then probing this with immune sera. This would confirm the results produced on probing the recombinant proteins since 2D SDS-PAGE would separate the native protein into its individual portions.

3. The ultrastructural morphology of the ovaries should be assessed to confirm the histological findings.

4. To adequately statistically assess the ovarian effects, a larger study needs to be done which would have adequate sample size for each point of ovariectomy.

5. Finally, the effects of these antibody populations on *in vitro* fertility should be assessed using the hemi-zona assay. In addition, the *in vivo* fertility should also be assessed using another study where ovariectomies would not be performed.

In conclusion, the studies reported in this thesis demonstrate the complexity of the ZP macromolecules as immunogens and antigens. They also emphasize the importance of determining which antigenic determinants of individual ZP molecules may be targeted to elicit antibodies that will inhibit fertilization but will not alter ovarian function, which is a pre-requisite for a safe human-based

immunocontraceptive. Indeed, current efforts in ZP-based immunocontraception are now focused on identifying or developing such antigens. For future studies the following salient points should be kept in mind.

1. Use of the ZP for immunocontraceptive purposes will require exact knowledge of the molecular structure and fertilization function of the constituent ZP glycoproteins. The amino acid sequences of the three ZP glycoproteins of various mammalian species as derived from their cloned cDNA sequences is already known. However, knowledge of the O- and N-linked glycan structures of the ZP is incomplete.

2. Further progress in this field will be heavily dependent on genetic engineering techniques not only because of the possibility of producing an adequate supply of the antigen at low cost but also because of the possibility of separating the epitopes involved in ovarian dysfunction and inhibition of fertility.

3. Since recombinant peptides result in low immunogenicity, it will also be important to look for ways in which the immunogenic response to these antigens can be enhanced by using such systems as the baculovirus and promiscuous tetanus toxoid epitopes.

4. The formation of a central data base of all known ZP experiments is imperative in order to determine future experiments. After evaluation of

this database, all possible types of regimen (including adjuvant formulations) to optimize the efficacy of this potential human-based immunocontraceptive should be determined. This pathway is important because the dissimilarities observed in various ZP-based studies are due to a) species differences in the antigenic determinants of zonae and/or the immunological response of the animals immunized; b) differences in amounts of antigen used for injection; or c) differences in the immunization methods used.

Whatever the future of such studies, the ZP antigen has however, found a place as a sterilization vaccine for animals. However, the control of world wide population growth still remains one of the most urgent problems of mankind. If all the people of the world are to enjoy the highest possible level of health and basic human rights, it is imperative that research on contraceptive development continues unhindered.

REFERENCES

Aitken, R. J., Richardson, D. W. and Hulme, M. (1984) Immunological interference with the properties of the zona pellucida. In: *Immunological aspects of reproduction in mammals.* (*Edited by Crighton, D. B.*) Butterworths, London. Pp 305-325.

Aitken, R. J., Paterson, M. and Thillai-Koothan, P. (1993) Contraceptive vaccines. *British Medical Bulletin* 49, 88-99.

Aitken, R. J., Paterson, M. and van Duin, M. (1996) The potential of the zona pellucida as a target for immunocontraception. *American Journal of Reproductive Immunology* 35, 175-180.

Alexander, N. J. and Isahakia, M. (1983) Sperm antigens related to infertility. In: *Immunology of reproduction.* (*Edited by Wegmann, T. G. and Gill, J. J.*) Oxford University Press, New York. Pp 43-60.

Anderson, D. J. (1983) Factors affecting the generation and contraceptive effectiveness of immune responses to sperm. In: *Immunology of reproduction.* (*Edited by Wegmann, T. G. and Gill, J. J.*) Oxford University Press, New York. Pp 209-218.

Bagavant, H., Yurewicz, E. C., Sacco, A. G., Talwar, G. P. and Gupta, S. K. (1993) Delineation of epitopes on porcine zona pellucida relevant for binding of sperm to oocyte using monoclonal antibodies. *Journal of Reproductive Immunology* 23, 265-279.

Bagavant, H., Thillai-Koothan, P., Sharma, M. G., Talwar, G. P. and Gupta, S. K. (1994) Antifertility effects of porcine zona pellucida-3 immunization using permissible adjuvants in female bonnet monkeys (*Macaca radiata*): reversibility, effect on follicular development and hormonal profiles. *Journal of Reproduction and Fertility* 102, 17-25.

Baker, T. G. (1987) Oogenesis and ovulation. In: *Reproduction in mammals : 1 Germ cells and fertilization*, 2nd edition. (*Edited by Austin, C. R. and Short, R. V.*) Cambridge University Press, Cambridge. Pp 17-45.

Bambra, C. S. (1993) Introduction. In: *Veterinary management and research techniques for reproductive studies in the baboon; a practical approach*. The Institute of Primate Research, National Museums of Kenya, Nairobi. Pp 78.

Bedford, J. M. (1994) The contraceptive potential of fertilization: a physiological perspective. In: *New concepts in fertility control*. (*Edited by Edwards, R. G.*) Oxford University Press, Oxford. Pp 52-72.

Berger, T., Davis, A., Wardrip, N. J. and Hedrick, J. L. (1989) Sperm binding to the pig zona pellucida and inhibition of binding by solubilized components of the zona pellucida. *Journal of Reproduction and Fertility* 86, 559-565.

Bleil, J. D. and Wassarman, P. M. (1985) Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Developmental Biology* 95, 317-324.

Bleil, J. D. and Wassarman, P. M. (1988) Galactose at the nonreducing terminus of O-linked oligosaccharides of mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity. *Cell Biology* 85, 6778-6782.

Brown, C. R. and Jones, R. (1987) Binding of zona pellucida proteins to a boar sperm polypeptide of M_r 53,000 and identification of zona moieties involved. *Development* 99, 333-339.

Dirnhofer, S., Wick, G. and Berger, P. (1994) The suitability of human chorionic gonadotrophin (hCG)-based birth-control vaccines. *Immunology Today* 15, 469-474.

Drell, D. and Dunbar, B. S. (1984) Monoclonal antibodies to rabbit and pig zonae pellucidae differentiate species cross-reactive and unique antigenic determinants. *Biology of Reproduction* 30, 435-444.

Drell, D., Wood, D., Bundman, D. and Dunbar, B. S. (1984) Comparison of the immunological response in rats and rabbits to porcine zona pellucida. B*iology of Reproduction* 30, 445-457.

Dunbar, B. S. (1987) Two- dimensional electrophoresis and immunological techniques. Plenum Press, New York. Pp 379.

Dunbar, B. S. and Raynor, B. D. (1980) Immunochemical characterization of porcine zona pellucida antigens. *Biology of Reproduction* 22, 941-954.

Dunbar, B. S. and Schwoebel, E. D. (1988) Fertility studies for the benefit of animals and human beings: development of improved sterilization and contraceptive methods. *Journal of American Veterinary Medical Association* 193, 1165-1170.

Dunbar, B. S. and Wolgemuth, D. J. (1984) Structure and function of the mammalian zona pellucida, a unique extra-cellular matrix. *Modern Cell Biology* 3, 77-111.

Dunbar, B. S., Wardrip, N. J. and Hedrick, J. L. (1980) Isolation, physicochemical properties and macromolecular composition of zona pellucida from porcine oocytes. *Biochemistry* 19, 356-365.

Dunbar, B. S., Timmons, T. M., Maresh, G. A., Niu, E. M. and Skinner, S. M. (1986) Species variation of zona pellucida antigens. *Journal of Reproductive Immunology* 6, 116-124.

Dunbar, B. S., Lo, C., Powell, J. and Stevens, V. C. (1989) Use of a synthetic peptide adjuvant for the immunization of baboons with denatured and deglycosylated pig zona pellucida glycoproteins. *Fertility and Sterility* 52, 311-318.

Dunbar, B. S., Avery, S., Lee, V., Prasad, S., Schwahn, D., Schwoebel, E., Skinner, S. and Wilkins, B. (1994) The mammalian zona pellucida: its biochemistry, immunochemistry, molecular biology and developmental expression. *Reproduction, Fertility and Development* 6, 331-347.

Eley, R. M. and Bambra, C. S. (1993) Reproductive physiology. In: *Veterinary management and research techniques for reproductive studies in the baboon: a practical approach.* (*Edited by Bambra, C. S.*) The Institute of Primate Research, National Museums of Kenya, Nairobi. Pp 29-48.

Epifano, O., Liang, L., Familari, M., Moos Jr., M. C. and Dean, J. (1995) Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development* 121, 1947-1956.

Griffin, P. D. (1983) A fertility regulating vaccine based on the carboxyl-terminal peptide of the β-subunit of human chorionic gonadotropin. In: *Immunology of reproduction.* (*Edited by Wegmann, T. G. and Gill, J. J.*) Oxford University Press, New York. Pp 43-60.

Griffin, P. D. (1991) The WHO task force on vaccines for fertility regulation. Its formation, objectives and research activities. *Human Reproduction* 6, 166-172.

Griffin, P. D. (1996) The immunoregulation of fertility-changes in perspectives. *American Journal of Reproductive Immunology* 35, 140-147.

Gulyas, B. J., Gwatkin, R. B. L. and Yuan, L. C. (1983) Active immunization of Cynomolgus monkeys (*Macaca fascicularis*) with porcine zona pellucida. *Gamete Research* 4, 299-307.

Hasegawa, A., Koyama, K. and Isojima, S. (1988) Immunogenicity of a 92-kDa component of porcine zona pellucida isolated using a monoclonal antibody (3A4-2G1) exclusively cross-reactive with porcine

and human zonae pellucidae. *Journal of Reproductive Immunology* 14, 73-82.

Hausfater, G. (1974) Estrous females; their effects on the social organization of the baboon group. In: *Proceedings from the symposia of the fifth congress of the International Primatological Society.* Nagoya, Japan, August 1974. Pp 117-128.

Henderson, C. J., Braude, P. and Aitken, R. J. (1987a) Polyclonal antibodies to a 32-kDa deglycosylated polypeptide from porcine zonae pellucidae will prevent human gamete interaction *in vitro*. *Gamete Research* 18, 251-265.

Henderson, C. J., Hulme, M. J. and Aitken, J. R. (1987b) Analysis of the biological properties of antibodies raised against intact and deglycosylated porcine zonae pellucidae. *Gamete Research* 16, 323-341.

Hendrickx, A. G. (1971) Embryology of the baboon. The University of Chicago Press, Chicago. Pp 205.

Hinsch, K-D., Hinsch, E., Meinecke, B., Topfer-Petersen, E., Pfisterer, S. and Schill, W-B. (1994) Identification of mouse ZP3 protein in mammalian oocytes with antisera against synthetic ZP3 peptides. *Biology of Reproduction* 51, 193-204.

Holmes, D. D. (1984) Clinical laboratory animal medicine; an introduction. Iowa State University Press, Ames, Iowa. Pp 125.

Jones, G. R., Sacco, A. G., Subramanian, M. G., Kruger, M., Zhang, S., Yurewicz, E. C. and Moghissi, K. S. (1992) Histology of ovaries of female rabbits immunized with deglycosylated zona pellucida macromolecules of pigs. *Journal of Reproduction and Fertility* 95, 513-525.

Jones, W. R. (1994) Gamete immunology. *Human Reproduction* 9, 828-841.

Khanna, J., Van Look, P. F. A and Griffin, P. D. (1994) Challenges in reproductive health research: biennial report, 1992-1993. World Health Organization. Pp 202.

Kirkpatrick, J. F. and Turner, J. W. (1991) Reversible contraception in non-domestic animals. *Journal of Zoo and Wildlife Medicine* 22, 392-408.

Kirkpatrick, J. F., Liu, I. M. K., Turner, J. W. and Bernoco, M. (1991) Antigen recognition in feral mares previously immunized with porcine zonae pellucidae. *Journal of Reproduction and Fertility Supplement* 44, 321-325.

Kirkpatrick, J. F., Liu, I. M. K., Turner Jr., J. W., Naugle, R. and Keiper, R. (1992) Long-term effects of porcine zonae pellucidae immunocontraception

on ovarian function in feral horses (*Equus caballus*). *Journal of Reproduction and Fertility* 94, 437-444.

Koyama, K., Hasegawa, A., Tsuji, Y. and Isojima, S. (1985) Production and characterization of monoclonal antibodies to cross-reactive antigens of human and porcine zonae pellucidae. *Journal of Reproductive Immunology* 7, 187-198.

Lee, V. H., Schwoebel, E., Prasad, S., Cheung, P., Timmons, T. M., Cook, R. and Dunbar, B. S. (1993) Identification and structural characterization of the 75-kDa rabbit zona pellucida protein. *Journal of Biological Chemistry* 268, 12412-12417.

Lincoln, D. W. (1992) Human contraception: development of new scientific opportunities. *Journal of Reproduction and Fertility Supplement* 45, 175-192.

Liu, I. K. M. and Shivers, C. A. (1982) Antibodies to the zona pellucida in mares. *Journal of Reproduction and Fertility Supplement* 32, 309-313.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R J. (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* 193, 265-275.

Mahi-Brown, C. A., Huang Jr., T. T. F. and Yanagimachi, R. (1982) Infertility in bitches induced by active immunization with porcine zonae pellucidae. *Journal of Experimental Zoology* 222, 89-95.

Mahi-Brown, C. A., Yanagimachi, R., Hoffman, J. C. and Huang, Jr. T. T. F. (1985) Fertility control in the bitch by active immunization with porcine zonae pellucida. Use of different adjuvants and patterns of estradiol and progesterone levels in estrous cycles. *Biology of Reproduction* 32, 761-772.

Mahi-Brown, C. A., McGuinness, R. P. and Moran, F. (1992) The cellular immune response to immunization with zona pellucida antigens. *Journal of Reproductive Immunology* 21, 29-35.

Maresh, G. A. and Dunbar, B. S. (1987) Antigenic comparison of five species of mammalian zonae pellucidae. *Journal of Experimental Zoology* 244, 299-307.

Maresh, G. A., Timmons, T. M. and Dunbar, B. S. (1990) Effects of extracellular matrix on the expression of specific ovarian components. *Biology of Reproduction* 43, 965-976.

Millar, S. E., Chamow, S. M., Baur, A. W., Oliver, C., Robey, F. and Dean, J. (1989) Vaccination with a synthetic zona pellucida peptide produces long term contraception in female mice. *Science* 246, 935-938.

Moller, C. C., Bleil, J. D., Kinloch, R. A. and Wassarman, P. M. (1990) Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. *Developmental Biology* 137, 276-286.

Mortillo, S. and Wassarman, P. M. (1991) Differential binding of gold-labeled zona pellucida glycoproteins mZP2 and mZP3 to mouse sperm membrane compartments. *Development* 113, 141-149.

Oikawa, T. and Yanagimachi, R. (1975) Block of hamster fertilization by anti-ovary antibody. *Journal of Reproduction and Fertility* 45, 487-494.

O'Rand, M. G. (1988) Sperm-egg recognition and barriers to inter-species fertilization. *Gamete Research* 19, 315-328.

Ownby, C. L. and Shivers, C. A. (1972) Antigens of the hamster ovary and effects of anti-ovary serum on eggs. *Biology of Reproduction* 6, 310-318.

Paterson, M., Thillai-Koothan, P., Morris, K. D., O'Byrne, K. T., Braude, P., Williams, A. and Aitken, R. J. (1992) Analysis of the contraceptive

potential of antibodies against native and deglycosylated porcine ZP3 *in vivo* and *in vitro*. *Biology of Reproduction* 46, 523-534.

Philips, D. M. and Shalgi, R. (1980) Surface architecture of the mouse and hamster ZP and oocyte. *Journal of Ultrastructural Research* 72, 1-12.

Prasad, S. V., Mujtaba, S., Lee, V. H. and Dunbar B . S. (1995) Immunogenicity enhancement of recombinant rabbit 55-kilodalton zona pellucida protein expressed using the baculovirus expression system. *Biology of Reproduction* 52, 1167-1178.

Raj, H. G. M. (1983) Effects of active immunization with follicle stimulating hormone (FSH) on spermatogenesis in the adult crab eating monkey- evaluation for male contraception. In: *Immunology of reproduction*. (*Edited by Wegmann, T. G. and Gill, J. J.*) Oxford University Press, New York. Pp 115-124.

Rhim, S. H., Millar, S. E., Robey, F., Luo, A-M., Lou, Y-H., Yule, T., Allen, P., Dean, J. and Tung, K. S. K. (1992) Autoimmune disease of the ovary induced by a ZP3 peptide from the mouse zona pellucida. *Journal of Clinical Investigation* 89, 28-35.

Ringuette, M. J., Sobieski, D. A., Chamow, S. M. and Dean, J. (1986) Oocyte-specific gene expression: molecular characterization of a cDNA coding for ZP-3, the sperm receptor of the mouse zona pellucida. *Proceedings of the National Academy of Sciences, USA*, 83, 4341-4345.

Sacco, A. G. (1977) Antigenic cross-reactivity between human and pig zona pellucida. *Biology of Reproduction* 16, 164-173.

Sacco, A. G. and Palm, V. S. (1977) Heteroimmunization with isolated pig zonae pellucidae. *Journal of Reproduction and Fertility* 51, 165-168

Sacco, A. G., Subramanian, M. G., Yurewicz, E. C., DeMayo, F. J. and Dukelow, W. R. (1983a) Heteroimmunization of squirrel monkeys (*Saimiri sciureus*) with a purified porcine zonae antigen (PPZA). Immune response and biological activity of antisera. *Fertility and Sterility* 39, 350-358.

Sacco, A. G., Yurewicz, E. C. and Zhang, S. (1983b) Immunoelectrophoretic analysis of the porcine zona pellucida. *Journal of Reproduction and Fertility* 68, 21-31.

Sacco, A. G., Subramanian, M. G., Yurewicz, E. C., Pierce, D. L. and Dukelow, W. R. (1986a) Antigenic profile and impact of immunization with zona pellucida antigens in primates. In: *Immunological approaches to*

contraception and promotion of fertility. (*Edited by Talwar, G. P.*) Plenum Publishing Corporation, New York. Pp 407-427.

Sacco, A. G., Yurewicz, E. C. and Subramanian, M. G. (1986b) Carbohydrate influences the immunogenic and antigenic characteristics of the ZP3 macromolecule (M_r 55,000) of the pig zona pellucida. *Journal of Reproduction and Fertility* 76, 575-586.

Sacco, A. G., Pierce, D. L., Subramanian, M. G., Yurewicz, E. C. and Dukelow, W. R. (1987) Ovaries remain functional in squirrel monkeys (*Saimiri sciureus*) immunized with porcine zona pellucida 55,000 macromolecule. *Biology of Reproduction* 36, 481-490.

Sacco, A. G., Yurewicz, E. C. and Subramanian, M. G. (1989) Effect of varying dosages and adjuvants on antibody response in squirrel monkeys (*Saimiri sciureus*) immunized with the porcine zona pellucida M_r= 55,00 glycoprotein (ZP3). *American Journal of Reproductive Immunology* 24, 1-8.

Saling, P. M. (1991) How the egg regulates sperm function during gamete interaction: facts and fantasies. *Biology of Reproduction* 44, 246-251.

Schwoebel, E., Prasad, S., Timmons, T., Cook, R., Kimura, H., Niu, E-M., Cheung, P., Skinner, S., Avery, S. E., Wilkins, B. and Dunbar, B. (1991)

Isolation and characterization of a full-length cDNA encoding the 55-kDa rabbit zona pellucida protein. *Journal of Biological Chemistry* 266, 7214-7219.

Schwoebel, E. D, VandeVoort, C. A., Lee, V. H., Lo, Y. K. and Dunbar, B. S. (1992) Molecular analysis of the antigenicity and immunogenicity of recombinant zona pellucida antigens in a primate model. *Biology of Reproduction* 47, 857-865.

Senanayake, P. (1994) Contraception by the end of the 20th century- the role of voluntary organizations. *Human Reproduction* 9, 1088-1096.

Shabanowitz, R. B. (1990) Mouse antibodies to human zona pellucida: evidence that human ZP3 is strongly immunogenic and contains two distinct isomer chains. *Biology of Reproduction* 43, 260-270.

Shivers, C. A. and Dunbar, B. S. (1977) Autoantibodies to zona pellucida: a possible cause for infertility in women. *Science* 197, 1082-1084.

Shur, B. D. and Hall, N. G. (1982) A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. *Journal of Cell Biology* 95, 574-579.

Skinner, S. and Dunbar, B. (1992) Localization of a carbohydrate antigenic determinant shared between oocytes and ovarian surface epithelium. *Journal of Histochemistry and Cytochemistry* 40, 1031-1036.

Skinner, S. M., Mills, T., Kirchick, H. J. and Dunbar, B. S. (1984) Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion. *Endocrinology* 115, 2418-2432.

Skinner, S. M., Timmons, T. M., Schwoebel, E. D. and Dunbar, B. S. (1990) The role of zona pellucida antigens in fertility and infertility. *Immunology and Allergy Clinics of North America* 10, 185-195.

Skinner, S. M., Prasad, S. V., Ndolo, T. M. and Dunbar, B. S. (1996) Zona pellucida antigens: targets for contraceptive vaccines. *American Journal of Reproductive Immunology* 35, 163-174.

Skutelsky, E., Ranen, E. and Shalgi, R. (1994) Variations in the distribution of sugar residues in the zona pellucida as possible species-specific determinants of mammalian oocytes. *Journal of Reproduction and Fertility* 100, 35-41.

Spira, A. (1994) Contraception by the end of the 20th century. *Human Reproduction* 9, 445-447.

Storey, B T. and Kopf, G. G. (1991) Fertilization in the mouse. In: *A comparative overview of mammalian fertilization.* (*Edited by Dunbar, B. S. and O'Rand, M. G.*) Plenum Press, New York. Pp 167-215.

Suleman, M. A. (1993) Baboon acquisition and quality assurance for reproductive studies. In: *Veterinary management and research techniques for reproductive studies in the baboon: a practical approach.* (Edited by Bambra, C. S.) The Institute of Primate Research, National Museums of Kenya, Nairobi. Pp 9-28.

Suzuki, H., Togashi, M., Adachi, J. and Toyoda, Y. (1995) Developmental ability of zona-free mouse embryos is influenced by cell association at the 4-cell stage. *Biology of Reproduction* 53, 78-83.

Timmons, T. M., Skinner, S. M. and Dunbar, B. S. (1990) Glycosylation and maturation of the mammalian zona pellucida. In: *Gamete interaction: prospects for immunocontraception.* (*Edited by Alexander, N., Griffin, D., Spieler, J. M. and Waites, G. M. H.*) Wiley-Liss, New York. Pp 277-292.

Trounson, A. O., Shivers, C. A., McMaster, R. and Lopata, A. (1980) Inhibition of sperm binding and fertilization of human ova by antibody to porcine zona pellucida and human sera. *Archives of Andrology* 4, 29-35.

Tung, K. S. K., Ang, J. and Lou, Y. (1996) ZP3 peptide vaccine that induces antibody and reversible infertility without autoimmune oophoritis. *American Journal of Reproductive Immunology* 35, 181-183.

Turner, J. W., Liu, I. K. M. and Kirkpatrick, J. F. (1992) Remotely delivered immunocontraception in captive white-tailed deer. *Journal of Wildlife Management* 56, 154-157.

Upadhyay, S. N., Thillai-Koothan, P., Bamezai, A., Jayaraman, S. and Talwar, G. P. (1989) Role of adjuvants in inhibitory influence of immunization with porcine zona pellucida antigen (ZP-3) on ovarian folliculogenesis in bonnet monkeys: a morphological study. *Biology of Reproduction* 41, 665-673.

Vagtborg, H. (1965) The baboon in medical research. In: *Proceedings of the first International Symposium on the baboon and its use as an experimental animal.* November, 5-8, 1963, San Antonio, Texas, USA. University of Texas Press, Austin. Pp. 275.

VandeVoort, C. A., Schwoebel, E. D. and Dunbar, B. S. (1995) Immunization of monkeys with recombinant complimentary deoxyribonucleic acid expressed zona pellucida proteins. *Fertility and Sterility* 64, 838-847.

Van Nest, G. A., Steimer, K. S., Haigwood, N. L., Burke, R. E. and Ott, G. (1992) Advanced adjuvant formulations for use with recombinant subunit vaccines. *Vaccines* 92, 57-62.

Vector laboratories, USA (1996) *Instructions for immunohistochemical staining using Vectastain® ABC kit.*

Wango, E. O. (1990) Non-human primates as models for research in human reproduction. *Discovery and Innovation* 2, 30-35.

Wassarman, P. M. (1988) The zona pellucida: a coat of many colours. *Bioessays* 6, 161-166.

Wolgemuth, D. J., Celenza, J., Bundman, D. S. and Dunbar, B. S. (1984) Formation of the rabbit zona pellucida and its relationship to ovarian follicular development. *Developmental Biology* 106, 1-14.

Wood, D. M., Liu, C. and Dunbar, B. S. (1981) Effect of alloimmunization and heteroimmunization with zonae pellucidae on fertility in rabbits. *Biology of Reproduction* 25, 439-450.

Yanagimachi, R. (1994) Mammalian fertilization. In: *The physiology of reproduction*, 2nd edition. (*Edited by Knobil, E. and Neill, J-D.*) Raven Press, Limited, New York. Pp 184-317.

Yonezawa, N., Hatanaka, Y., Takeyama, H. and Nakano, M. (1995) Binding of pig sperm receptor in the zona pellucida to the boar sperm acrosome. *Journal of Reproduction and Fertility* 103, 1-8.

APPENDICES

Appendix 1 Eley and Bambra's method for the evaluation of sex skin scores in female baboons

Stage 0: The perineal skin is flat, pale pink in colour, and heavy deep wrinkles of sex skin, especially around the rectum, are visible. There is no swelling of the vulval lips. This is the quiescent stage which lasts eight to ten days before merging into stage 1.

Stage 1: The vagina begins to enlarge with slight paravaginal swelling. The wrinkles of the skin begin to unfold stretching the skin slightly. The colour is dull pink. If the animal is viewed from the side, no swelling of the vaginal area is evident when the tail is raised. The stage lasts two to three days.

Stage 2: The vaginal swelling begins to approach the ischial callosities and the expansion is downward. Any wrinkles, if present , are not as deep as in stage 0 and the colour is a brighter pink than in stage 1. If the female is viewed from the side slight swelling of the vulva may be seen. This stage lasts about three to four days.

Stage 3: There is continued expansion and stretching of the skin downwards and outwards. The colour changes to a bright pink and then red. When viewed from the side there is a distinct swelling backwards of the vulval lips and the perineum. Duration is three to four days.

Stage 4: Wrinkles disappear completely and in some animals swelling is at a maximum, reaching as far outwards as the ischial callosities and downwards. From the side the swelling is prominent and red in colour. Duration is four to six days.

Stage 5: This stage is not attained in some animals; for their maximum turgescence has the characteristics of stage 4. In stage 5 the colour of the skin is deep red and shiny and there is an absolute absence of wrinkles. The sex skin is large and gives the impression of an over-inflated balloon. When viewed from the side the whole rear end of the animal appears inflated. Often, if the animal has been cycling frequently, the tautness of the skin is so extreme that cuts which bleed appear. The stage can last up to ten days and as noted above may not be present in all animals.

Some researchers assign stage 5 as the maximum inflation of all animals regardless of the extent of that inflation in relation to other animals. At IPR the preference is to combine the appearance with the designation such that in some animals, stage 5 may not be achieved and maximum inflation is stage 4 or even on rare occasions stage 3. Ovulation occurs in the last two or three days of the maximum inflation of an animal. Stage 1 to 5, beginning with menstruation and ending with deturgescence, are referred to as: inflationary stage; pre-ovulatory phase; the follicular phase; and the period of turgescence or the period of tumescence respectively. Records at IPR show that the follicular phase is relatively constant regardless of the cycle length while the luteal phase is variable.

Stage 6: This is the stage of detumescence and lasts up to ten days. Wrinkles begin to form on the sex skin and the expanded area begins to deflate. At the same time, there is a noticeable change in colour with the sex skin changing from bright red to a dull red. If the animal has been mated successfully this then may turn crimson. If not, stage 0 resumes.

Stage 7: This stage lasts one to three days and is when menstrual bleeding is obvious. The amount of bleeding varies from a few drops which may hardly be seen to heavy bleeding. The latter parts of this stage usually coincide with stage 1, that is stage of inflation.

Stage 8: This is the stage of pregnancy and is denoted by a completely flat perineum of crimson colour; the sex skin is often observed flaking off. This stage lasts approximately six months.

Stage 9: This is the period post-partum and prior to resumption of cycling. It is the period of lactational amenorrhea. The skin is flat and pinkish-grey in colour.

Appendix 2 Preparation of buffer for solubilizaton of antigens for immunization

The buffer used was sodium carbonate buffer, pH 9.6.

a) 0.1 M sodium carbonate monohydrate ($Na_2CO_3.H_2O$) [Sigma, USA]

1.24 g in 100 ml water (H_2O)

b) 0.1 M sodium bicarbonate ($NaHCO_3$) [Sigma, USA] 0.84 g in 100 ml H_2O

c) Add (a) to (b) while monitoring the pH until pH 9.6 is reached.

Appendix 3 Preparation of buffers, reagents, solutions and substrate for enzyme-linked immunosorbent assay

a) Antigen coating buffer (0.1 M sodium carbonate)

0.1 M $Na_2CO_3.H_2O$ (Sigma, USA) 1.24 g in 100 ml H_2O

0.1 M $NaHCO_3$ (Sigma, USA) 0.84 g in 100 ml H_2O

Add the first solution to the second one while monitoring the pH until pH 9.6 is reached.

b) Blocking solution (2% milk in antigen-coating buffer)

20 g instant non-fat dried milk (KCC, Kenya)

Bring to 1000 ml with antigen coating buffer and stir well until milk is completely dissolved. Use fresh or store at -20 °C for later use.

c) Assay buffer (20 mM phosphate buffered saline plus Tween 20)

230 ml of a 0.02 M sodium phosphate (NaH_2PO_4) [Kobian, Kenya]

2.78 g/litre H_2O

770 ml of a 0.02 M disodium phosphate heptahydrate ($Na_2HPO_4.7H_2O$) [Sigma, USA]

5.36 g/litre H_2O

150 mM sodium chloride (NaCl) [Sigma, USA]	8.18 g/litre H_2O
0.05% Tween 20 (BioRad, USA)	0.5 ml
pH 7.3	

d) Diluent for primary antibody (2% milk in assay buffer)

2% non-fat dried milk (KCC, Kenya)	2 g

Bring to 100 ml with assay buffer and store at -20° C

e) Substrate buffer (citrate/phosphate, pH 5)

0.05 M citric acid (Sigma, USA)	0.48 g in 50 ml H_2O
0.1 M $Na_2HPO_4.7H_2O$ (Sigma, USA)	2.68 g in 100 ml H_2O

Mix 24.3 ml of citric acid solution (solution 1) with 25.7 ml of Na_2HPO_4 (solution 2) and adjust pH to 5.0 with either solution. Store at -20° C.

f) Primary antibody (from serum of experimental animals) diluted in diluent buffer (made day assay is run).

g) Secondary antibody (peroxidase-conjugated rabbit IgG fraction to human IgG Fc, Organon Teknika, USA) diluted in assay buffer (made day assay is run).

h) Inactivating reagent (to be made fresh on the day assay is run).

100 μl 30% hydrogen peroxide (H_2O_2) [Sigma, USA] in 10 ml methanol (Sigma, USA).

i) Enzyme substrate solution (made fresh on day of assay)

Dissolve 8 mg of substrate o-Phenylenediamine dihydrochloride (OPD) [Sigma, USA] in 10 ml substrate buffer and add 100 μl H_2O_2 to 10 ml of this substrate mixture. Protect this substrate from light before use.

Appendix 4. Preparation of buffers, reagents, solutions and substrate for immunoblotting

a) Protein solubilization buffer

2% sodium dodecyl sulphate (SDS) [Biorad, USA]	2 g
0.0625M Trizma base (Fisher, USA)	0.75 g
10% glycerol (Sigma, USA)	10 ml

H_2O to 100 ml, pH 6.8. Add a few grains of bromophenol blue (Sigma, USA) to colour and filter using a sterile filter. This solution is stored at room temperature and only less than one month-old solutions are used.

b) Bis-acrylamide

30% acrylamide (BioRad, USA)	30.0 g
0.8% bis-acrylamide (BioRad, USA)	0.8 g

H_2O to 100 ml. Filter using a 0.2 μm sterile filter and store at 4 °C.

c) Running gel buffer

1.5 M Trizma base (Fisher, USA) 18.2 g

0.4% SDS (BioRad, USA) 0.4 g

H_2O to 100 ml, pH 8.8 and store at 4 °C.

d) 10% ammonium persulfate (BioRad, USA)

1g ammonium persulfate in 10 ml of H_2O; aliquot into smaller volumes and store frozen. Aliquot in current use stored at 4 °C.

Various percentages of running gels can be made depending on the relative amounts of the various solutions constituting the gels as outlined in Table 8 below.

Table 8 Constituents for various percentages of running gels

	7.5 %	10 %	12.5 %	15 %	20 %
Running gel buffer	2.61 ml	2.61 ml	2.61 ml	2.61 ml	2.61 ml
Bis-acrylamide	2.63 ml	3.50 ml	4.38 ml	5.25 ml	6.99 ml
Distilled water	5.22 ml	4.35 ml	3.47 ml	2.60 ml	0.86 ml
TEMED	4.8 μl	4.8 μl	4.8 μl	4.8 μl	4.8 μl
10 % APS	39.8 μl	39.8 μl	39.8 μl	39.8 μl	39.8 μl

e) Water saturated sec-butanol

Butanol (Sigma, USA)	75 ml
H_2O	35 ml

Mix by shaking and allow to settle. Store at room temperature.

f) Stacking gel buffer

0.5M Trizma base (Fisher, USA)	30 g
0.4% SDS (BioRad, USA)	2 g

H_2O to 500 ml, pH 6.8 and store at 4 °C.

g) The reagents for the stacking gel are as follows.

Stacking gel buffer	1.26 ml
Bis-acrylamide	0.75 ml
Distilled water	3.01 ml
TEMED (BioRad, USA)	4.00 µl
10 % APS	24.00 µl

h) Tank buffer

0.025 M Trizma base (Fisher, USA)	15 g
0.192 M glycine (BioRad, USA)	72 g
0.1% SDS (BioRad, USA)	5 g

H_2O to 5 litres and store at room temperature.

i) Coomassie blue stain

50 % ethanol (Sigma, USA)	200 ml
45 % water	180 ml
5 % glacial acetic acid (Merck, Germany)	20 ml
0.2 % Coomassie® Brilliant Blue (BioRad, USA)	0.8 g

Filter after preparation and store at room temperature.

j) Transfer buffer

Trizma base (Fisher, USA)	1.16 g
Glycine (BioRad, USA)	0.58 g
10% SDS (BioRad, USA)	0.75 ml
Methanol (Sigma, USA)	20 ml
H_2O	100 ml

This solution is made fresh when needed.

k) Phosphate buffered saline (10X)

NaCl (Sigma, USA)	80 g
KCl (Potassium chloride) [Sigma, USA]	2 g
Na_2HPO_4 (Disodium phosphate) [Kobian, Kenya]	29 g
KH_2PO_4 (Potassium phosphate) [Sigma, USA]	2 g

Make to 1 litre with distilled water and pH to 7.2-7.4.

Dilute ten times with distilled water to make 1X PBS.

l) Blocking solution- 5% non-fat powdered milk

Milk (KCC, Kenya)	50 g
1X PBS	1 litre

Mix well using magnetic stirrer and use while fresh.

m) Primary antibody (from serum of experimental animals) diluted in blocking solution.

n) Secondary antibody (peroxidase-conjugated rabbit IgG fraction to human IgG Fc, Organon Teknika, USA) diluted in blocking solution.

o) 3'3 Diaminobenzidine tetrahydrochloride (DAB) [Sigma, USA] substrate solution

1X PBS	10 ml
Stock DAB (4% DAB or 40 mg/ml DAB. Stored at - 20 ^{o} C)	200 µl
8% Nickel Chloride ($NiCl_2$) [Sigma, USA]	50 µl
50% H_2O_2 (Sigma, USA)	2 µl

Appendix 5. Preparation of buffers, solutions and substrate for immunohistochemistry

a) 10X PBS as in appendix 4 (k).

b) 3% Bovine serum albumin (BSA) [Sigma, USA]

Dissolve 3g of BSA in 100 ml of 1X PBS.

c) 0.1% BSA

Take 33.3 ml of 3% BSA and add 1X PBS to 1 litre.

d) Primary antibody (from serum of experimental animals) diluted in 3% BSA.

e) Secondary antibody (peroxidase-conjugated rabbit IgG to human IgG Fc, Organon Teknika, USA) diluted in 3% BSA.

f) DAB substrate solution (made just before use).

1X PBS	10 ml
Stock DAB (as in appendix 4o)	87.5 µl
3% H_2O_2	3 µl

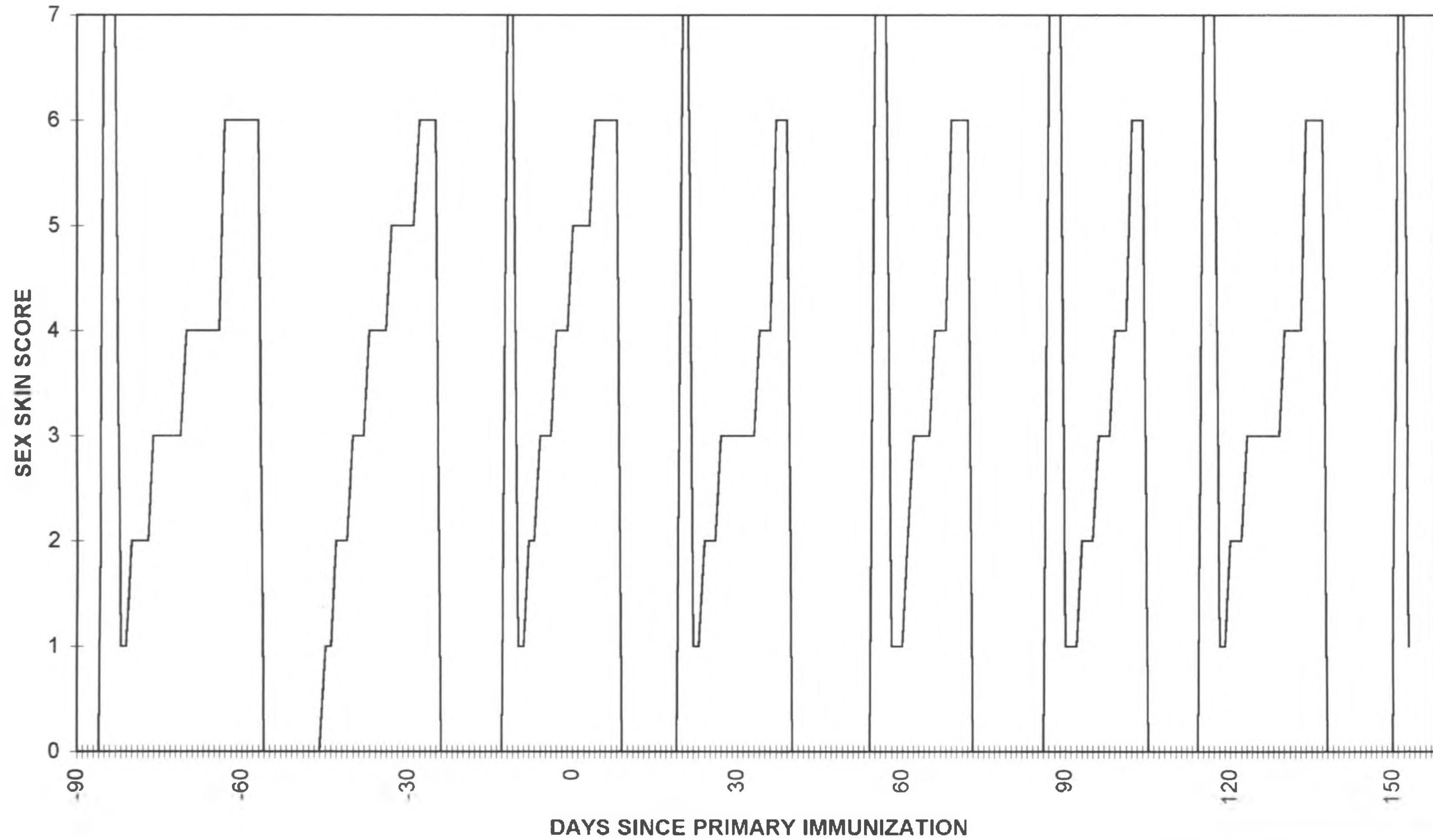
Mix well.

Appendix 6. Emulsification of zona pellucida antigens

TiterMax® adjuvant was used. For preparation of the solution for injection, a 12 cm length of emulsification tubing was used. Two 1½ inch 18 gauge needles (Becton Dickinson, USA) were threaded whole length into the tubing on either side. These needles were each then connected to a 3 cc Luerlok syringe (Becton Dickinson, USA). The TiterMax® was warmed to room temperature; then vortexed for 60 seconds to make sure that it was a homogeneous suspension of copolymer-coated microparticles.

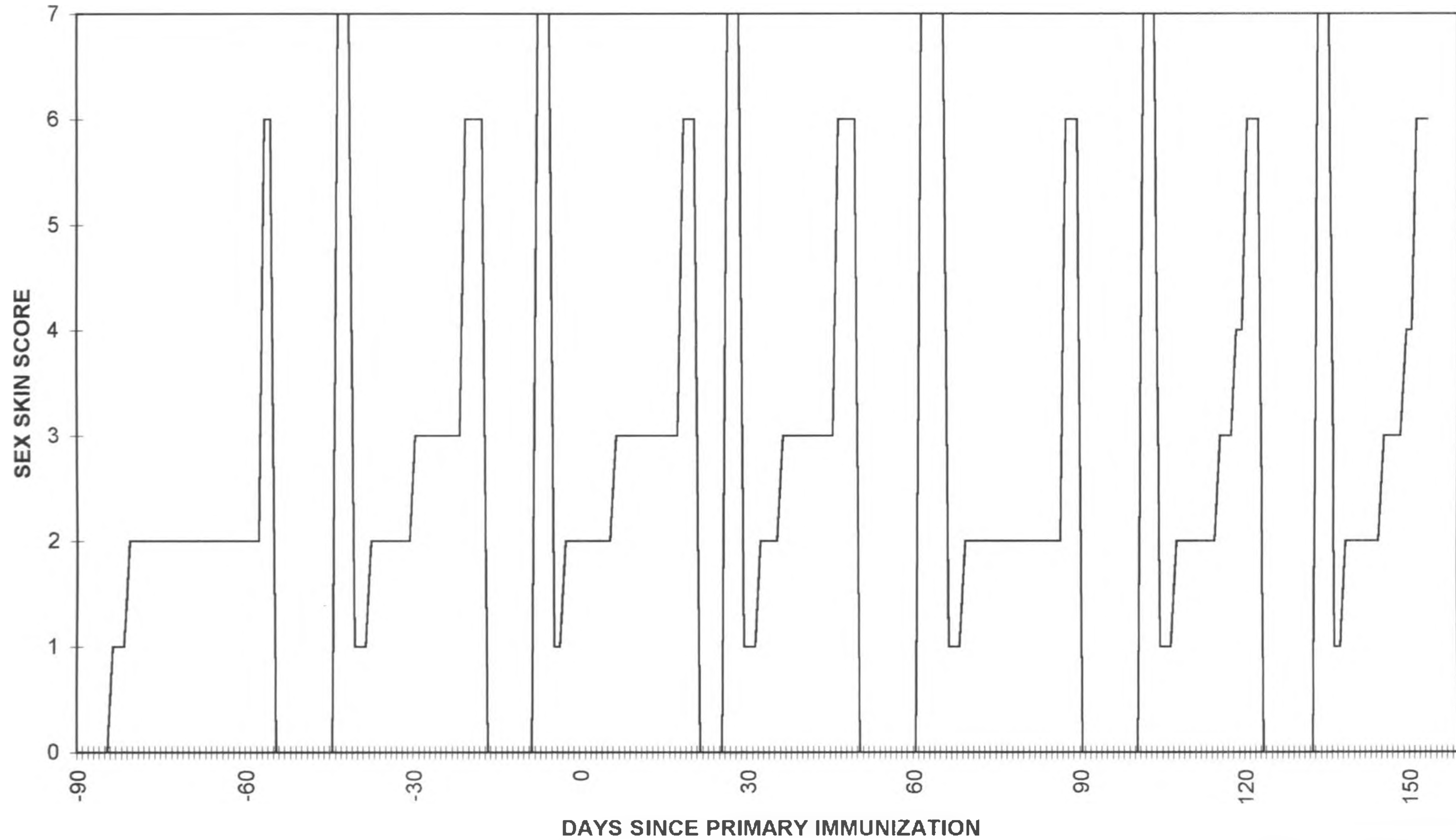
After vortexing, one syringe was loaded with TiterMax® and the other with half the volume of solubilized antigen (solubilization buffer only for the controls). It is important to add the aqueous antigen phase to the TiterMax® in at least two small volumes. The two solutions were then mixed by forcing the materials back and forth through the tubing until a whipped-cream-like water-in-oil emulsion was formed. It is also important to push the antigen into the TiterMax® syringe first, so that the aqueous phase enters the oil phase rather than vice versa. After the whipped-cream-like water-in-oil emulsion formed it was all pushed into one syringe and the empty one disconnected from its needle. This empty one was then loaded with the remaining half of the aqueous antigen solution. After reconnection of the syringe to the tubing, emulsification was then done until the required consistency was obtained. The emulsion was then pushed into one syringe and the full syringe disconnected from the emulsification tubing. This syringe was then loaded with a 21 gauge needle for injection. Different needles were used for each animal.

Appendix 7



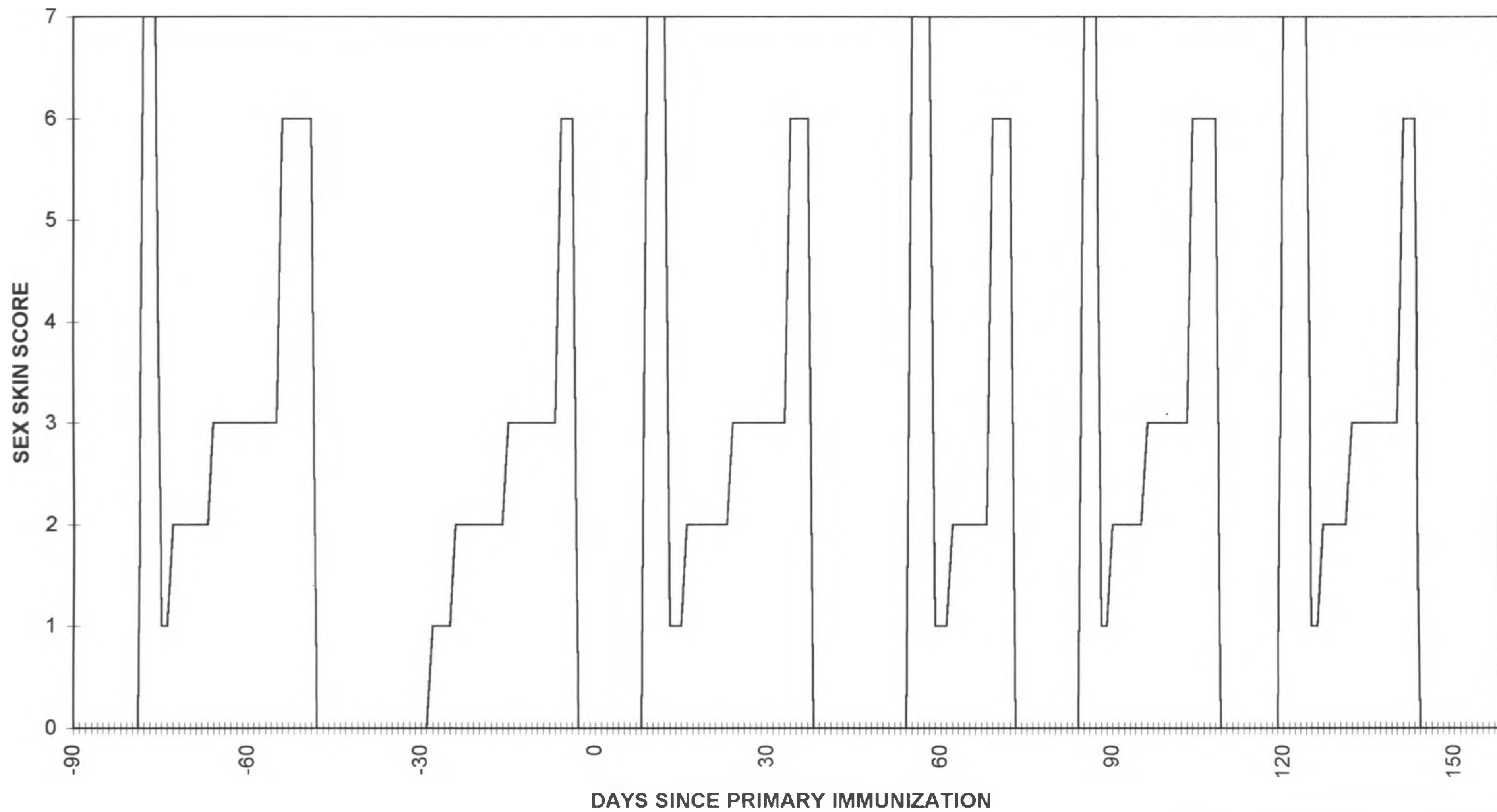
Appendix 8

CYCLE CHART FOR 2025 (CONTROL) BEFORE AND AFTER IMMUNIZATION



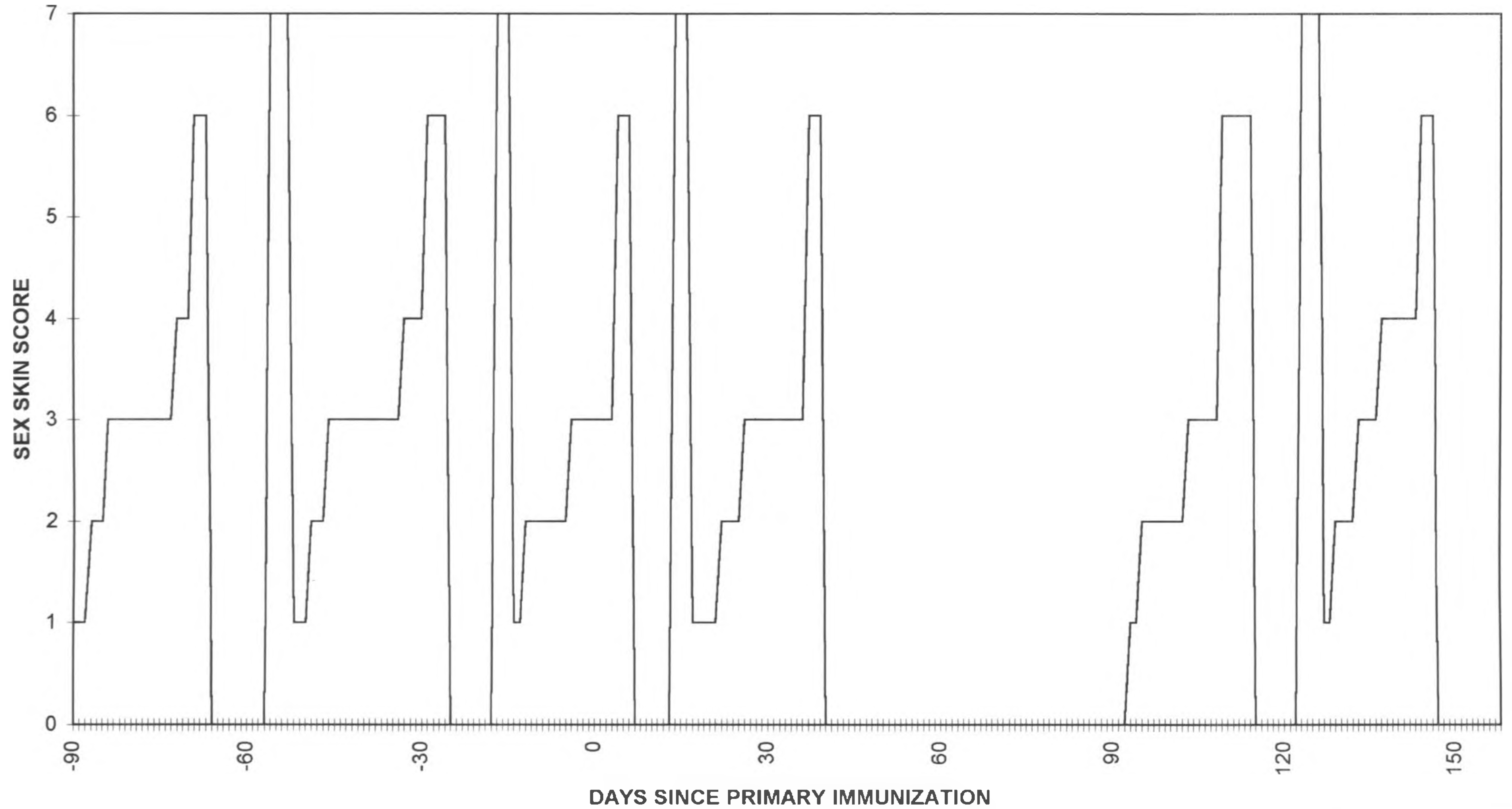
Appendix 9

CYCLE CHART FOR 2033 (CONTROL) BEFORE AND AFTER IMMUNIZATION

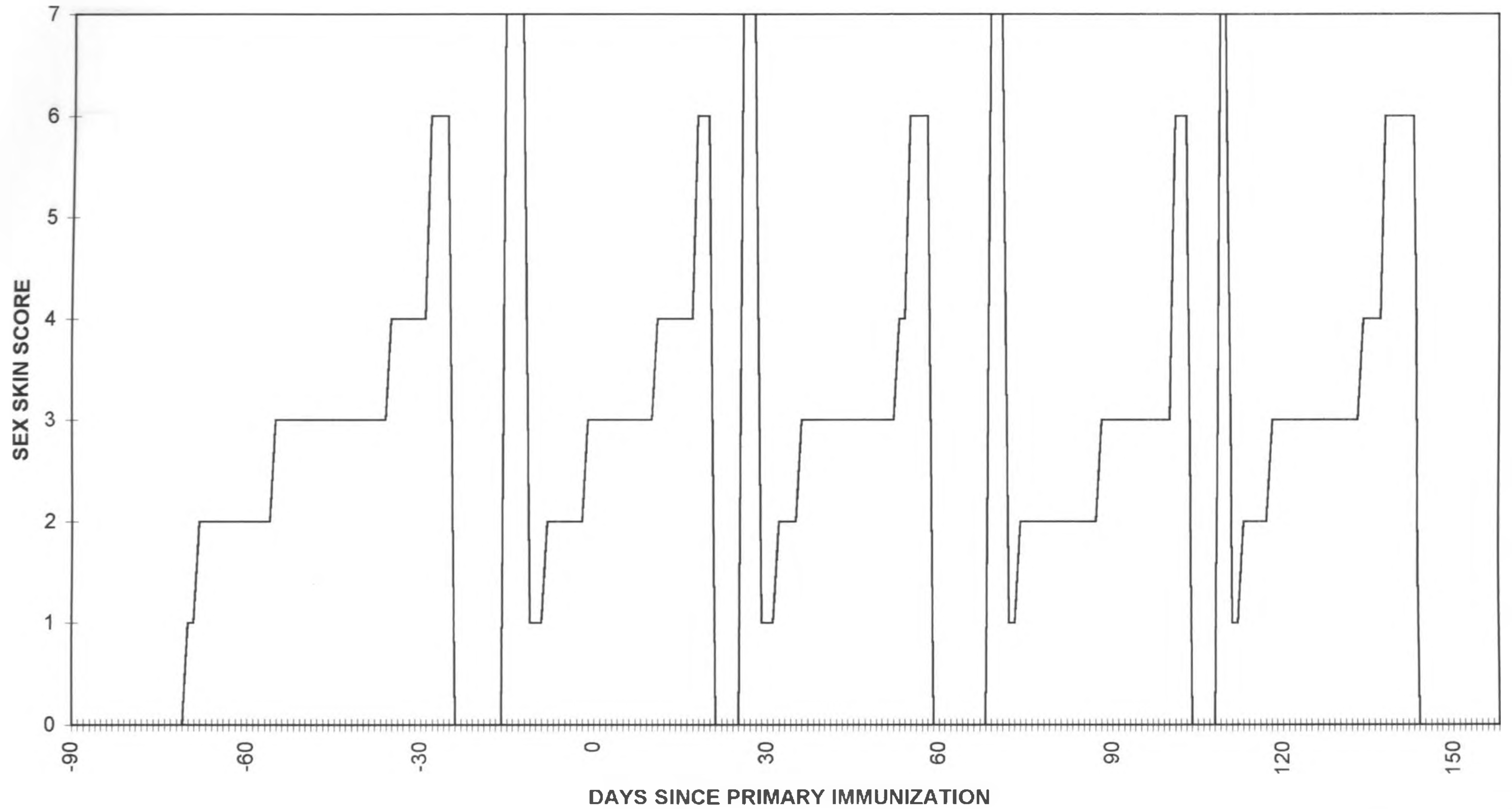


Appendix 10

CYCLE CHART FOR 1994 (PIG ZP) BEFORE AND AFTER IMMUNIZATION

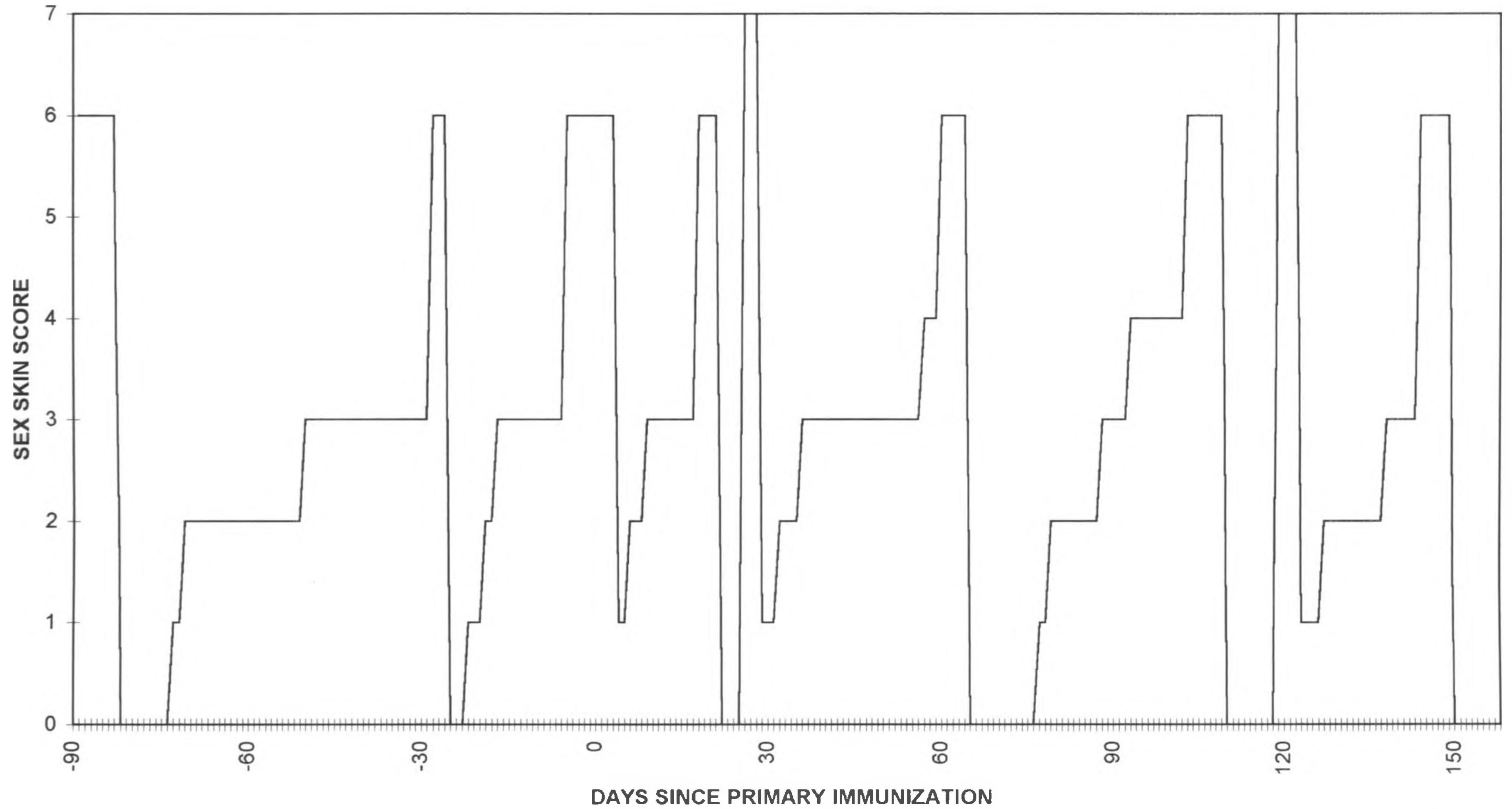


Appendix 11

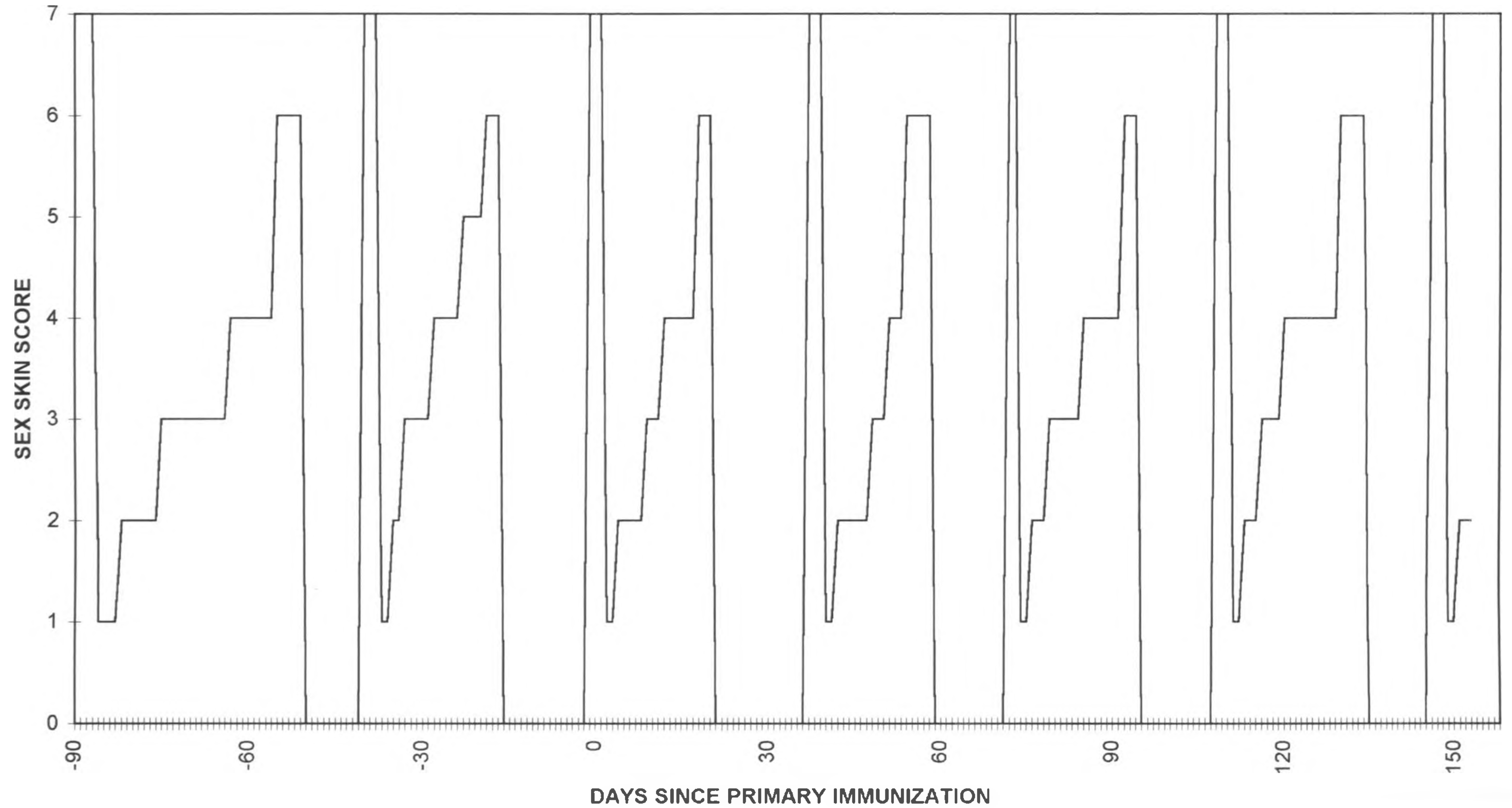


Appendix 12

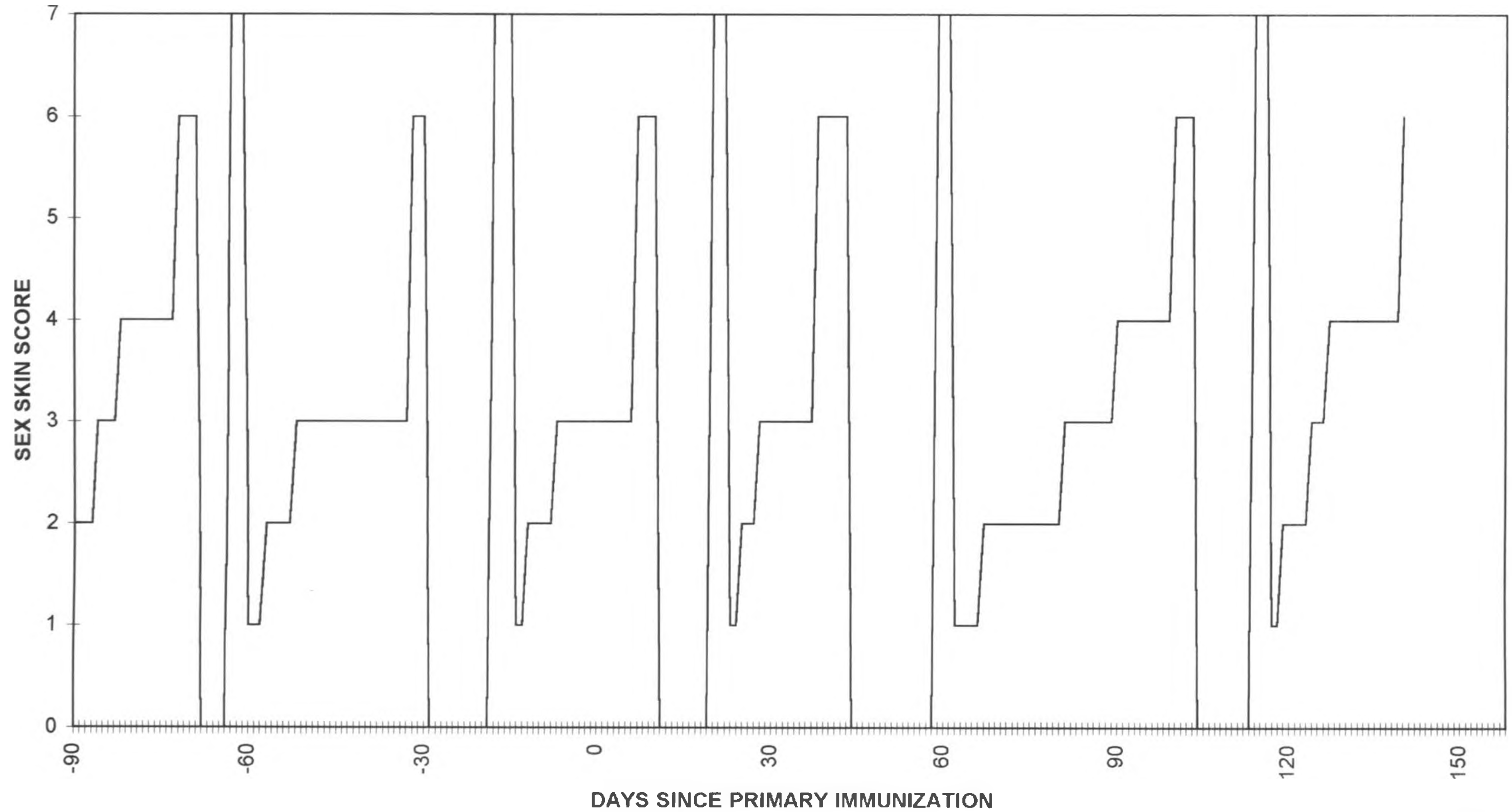
CYCLE CHART FOR 2032 (PIG ZP) BEFORE AND AFTER IMMUNIZATION



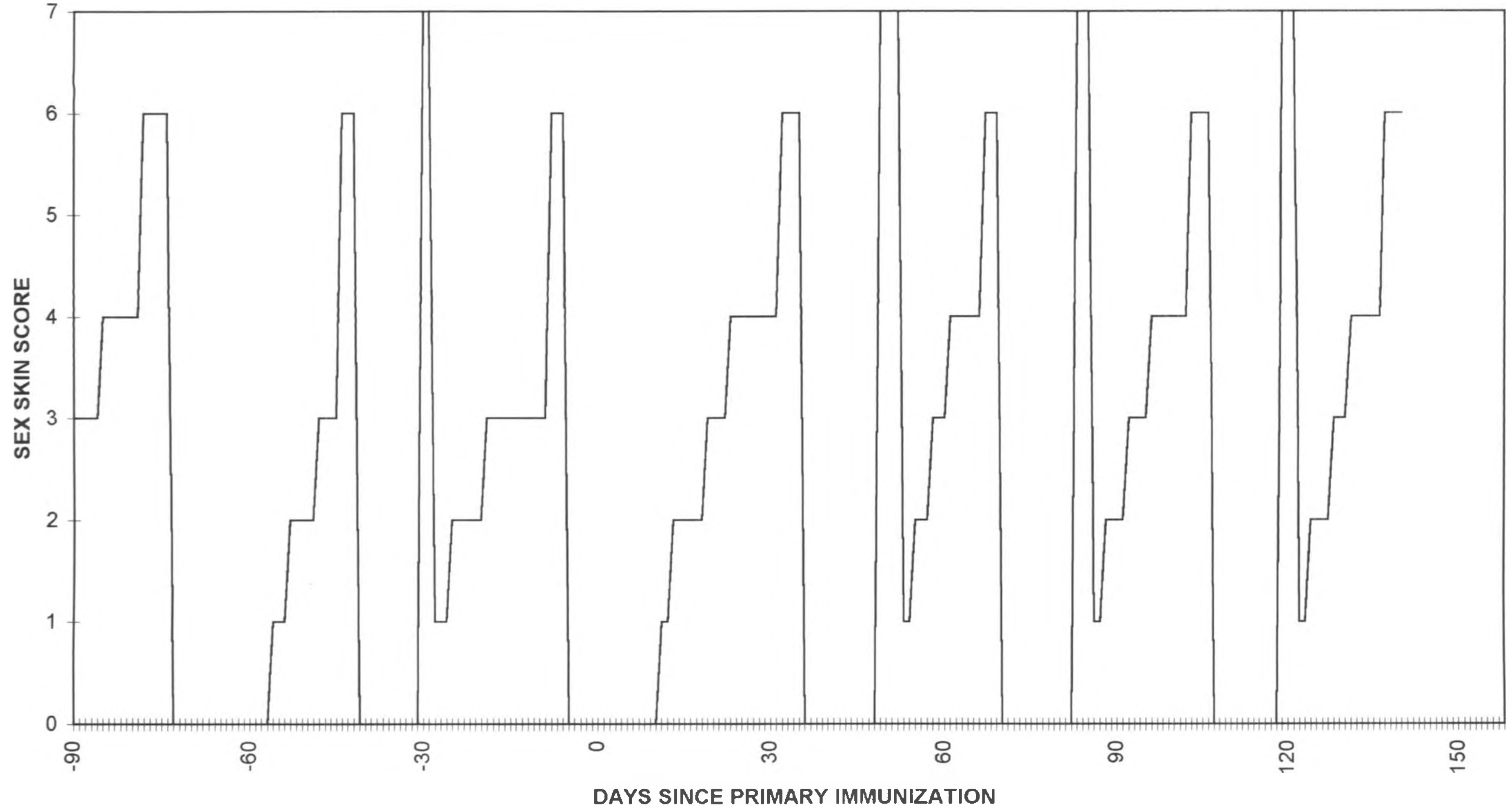
Appendix 13



Appendix 14

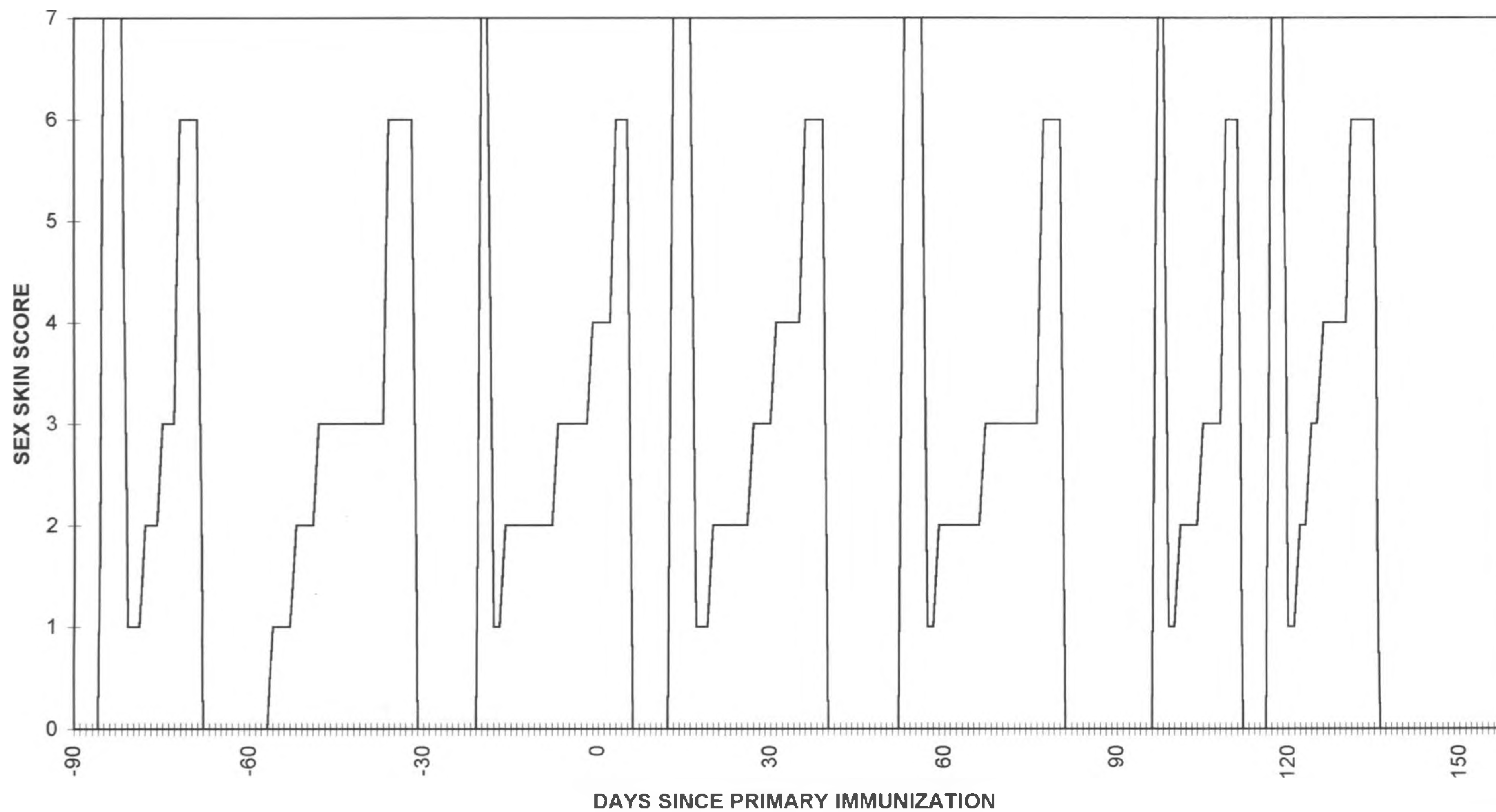


Appendix 15



Appendix 16

CYCLE CHART FOR 1997 (RABBIT ZP) BEFORE AND AFTER IMMUNIZATION



Appendix 17