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Analysis of Oocyte Quality in the Rhesus Macaque (*Macaca mulatta*)

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Analysis of Oocyte Quality in the Rhesus Macaque (*Macaca mulatta*)

A Dissertation

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Conservation Biology
Reproductive Biology

by

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B.S. College of William and Mary, 1997
M.S. San Jose State University, 2001

May, 2007

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Dedication

This dissertation is dedicated to my parents

Mr. John A. Nichols

and

Mrs. Gina G. Nichols

who have always given me love and support

throughout my education

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List of Abbreviations

AI = **A**rtificial **I**nsemination

ART = **A**ssisted **R**eproductive **T**echnologies

CG = **C**horionic **G**onadotropin

ET = **E**mbryo **T**ransfer

FSH = **F**ollicle **S**timulating **H**ormone

GV = **G**erminal **V**esicle

GVBD = **G**erminal **V**esicle **B**reakdown

IVF = *In Vitro* **F**ertilization

IVM = *In Vitro* **M**aturation

IVO = *In Vivo* **M**aturation

LH = **L**uteinizing **H**ormone

MII = **M**etaphase of **M**eiosis **II**

SSP = **S**pecies **S**urvival **P**lan

Abstract

Many primate populations face the threat of extinction due to habitat loss, intensive agriculture, hunting for meat, the pet trade and/or use in traditional medicines. An alternative approach to *in situ* conservation includes gene banking and the use of assisted reproductive technologies (ART), such as oocyte *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). Although many of these ‘high-tech’ solutions have not yet been proven viable for pragmatic wildlife conservation, basic research and development of these emerging tools can provide necessary information needed to optimize these techniques and institute ART as a routine practice in conservation efforts. A severely limiting factor in the successful application of ARTs is the availability of mature developmentally competent oocytes. Oocyte maturation involves many nuclear and cytoplasmic factors, which can be affected by maturation conditions and female age. *In vitro* maturation does not have the same success rate across species studied. In primates especially, IVM oocytes exhibit reduced developmental capacity upon fertilization when compared to *in vivo* matured (IVO) oocytes. This study aimed to investigate possible causes of reduced developmental capacity of primate IVM oocytes using the rhesus macaque (*Macaca mulatta*) as a model. Research efforts included investigation of ovarian senescence, oocyte karyotype and spindle morphology, and establishment of an optimal sperm cryopreservation protocol for use in IVF.

Histological examination of the rhesus ovary demonstrated an age-related pattern of follicle depletion similar to that described in the human ovary. Oocyte karyotype analysis revealed a significant effect of IVM on the frequency of hyperhaploidy. In addition, immunostaining and confocal microscopy demonstrated a significant increase of anomalous chromosome congression on the oocyte metaphase II spindle equator in relation to IVM and

donor female age. These results indicate that IVM can produce serious, if not lethal consequences for embryo development. This study presents baseline data on ovarian aging in the rhesus macaque and aspects of nuclear maturation during macaque IVM that may contribute to the design of primate oocyte recovery plans. Implementation of either of two sperm cryopreservation methods originally developed for rhesus and vervet monkeys will aid future investigation of the developmental capacity of IVM oocytes.

Keywords: assisted reproductive technologies, *in vitro* fertilization, *in vitro* maturation, oocyte karyotype, oocyte metaphase spindle morphology, ovarian senescence, rhesus macaque, sperm cryopreservation.

Chapter 1

Analysis of oocyte quality in the rhesus macaque (*Macaca mulatta*)

Application of Assisted Reproductive Technologies to endangered species

Many higher primates and all apes (gibbons (genus *Hylobates*); chimpanzee (*Pan* spp.); gorilla (*Gorilla*); and orangutan (*Pongo*)) are considered threatened or endangered by the Convention on International Trade in Endangered Species (CITES 2003). These animals face the threat of extinction mainly due to habitat loss, intensive agriculture, the pet trade, and, in some cases, hunting for meat and/or use in traditional medicines. These issues present major challenges for conservation and have been approached in several ways. The most obvious and direct approach is to enforce habitat preservation, create national reserve areas, and institute and enforce laws protecting these species from poaching. However, these methods often run into formidable economical, political and geographical issues. Choices made concerning local land use, resource management and the ever increasing human population and its demands dictate fates of ecosystems and present challenges for habitat preservation. Individual localities decide priorities of resource preservation and distribution, often to the detriment of the environment.

Due to depletion of wild animal populations, many zoos worldwide have established small groups of threatened or critically endangered species in order to study and preserve these animals. For numerous species, these small enclaves have come to represent a significant proportion of the world's total population. In an attempt to compensate for the hurdles that *in situ* conservation often presents, zoos have developed international captive breeding programs. Species Survival Plans (SSPs), which regulate breeding schedules by individuals of each species, have been established and have enjoyed some success. However, these plans may result in

genetic “bottlenecking” in captive species for which there is usually a low number of individuals available for breeding. Also, this method is not applicable to captive populations possessing individuals that may be diseased, infertile or simply not breeding in captivity. Another obstacle encountered in captive breeding programs is the exchange and transport of individual animals. Shipping animals is not desirable because this causes stress, can introduce disease and may be deleterious to reproductive activity. Transported individuals often require long periods of quarantine and time to acclimate to their new environment and counterparts before they will resume normal mating behavior, if ever.

Other avenues to address species conservation include gene banking and the use of assisted reproductive technologies (ART). These approaches cannot replace *in situ* conservation or captive breeding programs, but they do provide additional alternatives for species propagation. Assisted reproductive technologies include such techniques as artificial insemination, *in vitro* maturation of oocytes and *in vitro* fertilization (IVM/IVF), intracytoplasmic sperm injection, somatic cell nuclear transfer (“cloning”), gamete and embryo cryopreservation and embryo transfer. These techniques provide opportunities for both the immediate use and long-term banking of genetic material from species whose captive population is too low to support a successful SSP or from individuals who may be diseased, infertile, unwilling to mate under captive conditions, produce poor quality spermatozoa or cannot carry a pregnancy. Many of these situations are similar to problems in human infertility, and research done in humans can be applied to studies on endangered animals, particularly primates. In fact, many of the protocols used were first demonstrated in humans (Edwards *et al.*, 1969; Steptoe and Edwards, 1978) before being applied to nonhuman primates (Bavister *et al.*, 1983). Data resulting from research performed on species that are plentiful may be extrapolated to their closely related counterparts

that are threatened or endangered. In addition, transport of the material used in ART – gametes and embryos – is much easier, economical and safer than whole animal transportation. The successful application of ART to conservation biology thus holds great promise for the preservation of ecologically valuable species and will be the focus of this dissertation.

To date, the assisted reproductive biotechnology most commonly applied to conservation is artificial insemination (AI), a comparatively simple technique in which ejaculated semen/spermatozoa are inserted directly into a recipient female's vagina or uterus, depending on the species. The most remarkable success story using AI is perhaps that of the black-footed ferret (*Mustela nigripes*), a species thought to be extinct 26 years ago and whose total wild population was reportedly a mere 18 individuals when rediscovered. Efforts by the National Zoological Park's Conservation and Research Center (Front Royal, VA, USA) included AI in their quest to turn the tide of this species' future and today the population of the black-footed ferret is healthy and growing (Williams *et al.*, 1991; Miller *et al.*, 1996; Biggins *et al.*, 1997). Another noteworthy achievement is that involving the giant panda (*Ailuropoda melanoleuca*), a species notoriously difficult to breed in captivity. Several births at zoos and specialized panda research centers have been reported recently, due, in part, to the use of hormone monitoring and AI (Zheng *et al.*, 1997; Yan *et al.*, 2000). However, this relatively simple approach cannot be used in all endangered species, nor can it overcome all reproductive problems. In many species needing attention, spermatozoa and receptive females are not usually readily available. In instances where the male of the species has a low sperm count and/or poor sperm motility, more complex techniques such as intracytoplasmic sperm injection (Pukanzhenti *et al.*, 2006) may need to be utilized. Technologies involving production of embryos *in vitro* are not as commonly applied to endangered species as they are in laboratory and some domesticated animal species,

and so they have not yet achieved the same success rates. However, there have been a few success stories. In 2001, Loi and colleagues reported the production of offspring of the endangered mouflon sheep (*Ovis orientalis*) using a suite of ARTs (Loi *et al.*, 2001; Ptak *et al.*, 2002) including cross-species nuclear transfer and embryo transfer into surrogate domestic sheep species. Investigators at the Audubon Center for the Research of Endangered Species in New Orleans, Louisiana have also utilized a range of ARTs in the production of several non-domestic cat species (Pope, 2000; Gomez *et al.*, 2004; Pope *et al.*, 2006). Successes such as these have been due to considerable basic research on ‘model’ species (e.g., the domestic cat as an experimental model for wild felids).

Progress in developing applicable ARTs is slow because these techniques must be customized for each species. At present, a wide variety of technical and biological difficulties restricts the full exploitation of ARTs for conservation purposes (Pukazhenti *et al.*, 2006). A fundamental problem is the low level, and sometimes a complete lack, of knowledge about the reproductive strategies and physiological mechanisms used by different mammals, even within the same genus. Wildt *et al.* (2003) have pointed out that most of what we know about mammalian reproduction is derived from only 14 species (including human, domestic livestock and various laboratory species) which represent only 0.3% of all known mammalian species.

Literature is particularly sparse regarding applications of ART to endangered primates. There have been a few studies performed in great apes, specifically the western lowland gorilla (*Gorilla gorilla gorilla*), a subspecies commonly held by zoos and one that currently enjoys a successful SSP program, and the common chimpanzee (*Pan troglodytes*). These preliminary studies indicate that semen collection and cryopreservation in these species has achieved some success. *In vitro* fertilization has also been performed in these animals and a western lowland

gorilla was born as a result of using ARTs (Pope *et al.*, 1997). In contrast, there are no reports of any success in producing chimpanzees using ART. In addition to studies performed on great apes, Moreland *et al.* (2001) used the southern black howler monkey (*Alouatta caraya*) as a model for other *Alouatta* species considered threatened or endangered. The majority of ART studies in non-human primates, however, have been performed in macaques (Old World monkey, genus *Macaca*) and the common marmoset (New World monkey, *Callithrix jacchus*). These species are not threatened in their natural environments and are excellent models for other primates because they have been well studied and their reproductive biology is well-characterized. The rhesus macaque (*Macaca mulatta*) (Figure 1.1) will be the model species on which the following dissertation is based. This species has been intensively studied over several decades and is considered to be the best non-human primate model for a wide variety of biomedical and disease research. It is thus an excellent model for related macaque species that are endangered, such as the lion-tailed macaque (*Macaca silenus*) and Sulawesi crested macaque (*Macaca nigra*). In the former case, ARTs developed first for humans and then modified for macaque monkeys have been applied to produce IVF embryos and offspring (Cranfield *et al.*, 1989, 1990).

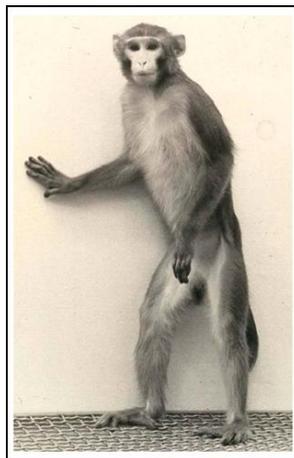


Figure 1.1. The first macaque, “Petri”, produced using IVF (Bavister *et al.*, 1984).

Assisted reproductive technologies rely, in general, on the availability of oocytes and/or spermatozoa. Acquisition of viable spermatozoa is not usually a problem. Spermatogenesis continues relatively undisturbed (except in seasonally breeding species) throughout the lifespan of the mature male, thereby ensuring an almost endless supply of gametes. On the other hand, oogenesis is completed prior to birth or soon afterwards in the female mammal. The primordial follicle population within an ovary therefore represents the entire reservoir of potentially viable oocytes available to the reproducing female. This population suffers dramatic decreases over the postnatal lifespan, partly due to follicular recruitment processes but mostly due to follicular atresia within the ovary. There is some evidence that oogenic stem cells may exist and continue to function past birth in the mouse (Johnson *et al.*, 2004). However, this has not been evident in other species and it should be noted that the presence of such cells did not stem the tide of follicular loss, only seeming to slow it down. Thus, the supply of oocytes is a limiting factor in studies using ART where embryos are produced *in vitro*.

Oocyte Maturation

Oocytes are not only limited in their availability, but are often developmentally immature upon collection. During its growth and development, the oocyte undergoes various nuclear and cytoplasmic changes, becoming a storehouse of molecules such as mRNA required for successful maturation, fertilization and embryonic development. Crucial to oocyte development is the proper timing and execution of meiosis in order to produce a mature oocyte receptive to sperm penetration. Two developmental arrests occur during this process (Figure 1.2) in the mammalian ovary, the first occurring during the diplotene stage of prophase during meiosis I. Oocytes at this stage are termed primary or germinal vesicle (GV) oocytes and remain arrested

until the onset of puberty after which they are periodically released from meiotic arrest when recruited into a follicular cohort. In this cohort, meiosis progresses until an additional arrest, in most mammals, occurs at metaphase of meiosis II. Morphological assessment of this stage, the “mature oocyte”, includes extrusion of the first polar body upon the completion of meiosis I. At this point, the primate oocyte is usually ovulated and can be fertilized.

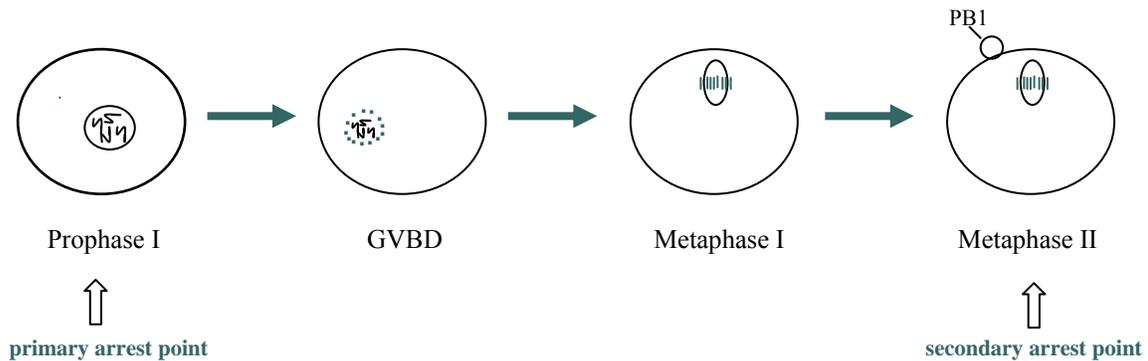


Figure 1.2. Arrest points during meiosis in the oocyte. Primary arrest occurs during the diplotene stage of prophase I and is released by hormonal stimulus (the “LH” surge). Once signaled to resume meiosis, the germinal vesicle undergoes breakdown (GVBD) and completion of meiosis I. Secondary arrest occurs at metaphase II and is morphologically distinguished by the presence of a polar body (“PB1” on the figure). The oocyte is said to have undergone “nuclear maturation” at this stage. At this point, ovulation in the macaque occurs followed by fertilization.

It is imperative for chromosomes to properly align and segregate during meiosis I and II. Failure to do so results in aneuploidy, i.e., an incorrect number of chromosomes, which is often fatal to embryo or fetal development. Metaphase spindle formation and chromosome segregation are believed to be particularly sensitive to both the physical and chemical environments (Albertini, 1992; Sanfins *et al.*, 2003), underscoring the important contribution of the maturation environment to the production of chromosomal aberrations.

Maturation conditions experienced by the oocyte can also affect patterns of gene expression during embryonic development as well as the capacity to develop into blastocysts (Knijn *et al.*, 2002; Krisher, 2004). Investigators utilizing ART have attempted to mimic *in vivo* conditions involved in oocyte maturation with limited success. Over 70 years ago, Pincus and Enzmann (1935) investigated the possibility of IVM of mammalian oocytes by using the rabbit as a model since these animals are induced ovulators and would, therefore, provide GV or immature oocytes for study. Results from these early studies demonstrated that mammalian oocytes were able to undergo spontaneous meiotic maturation *in vitro*. Later studies using IVF discovered that although oocytes underwent “nuclear maturation” (see Figure 1.2), they were not developmentally competent. That is, these oocytes did not consistently undergo normal embryonic development after fertilization (Thibault, 1977). It appeared that in addition to the nuclear changes the oocytes must undergo, cytoplasmic changes must also take place in order for the oocyte to become fully competent.

”Cytoplasmic maturation”, the term commonly used to refer to the processes that prepare the oocyte for activation, pronuclei formation, and preimplantation development (Eppig *et al.*, 1994), is poorly understood (Schramm and Bavister, 1999). Undoubtedly, the mechanisms driving cytoplasmic maturation include storage of maternal molecules, such as messenger RNA, and preparation for proper initiation and timely expression of these messages. Failure of cytoplasmic maturation can result in impairments in the transition of gene expression and transcription from maternal to embryonic sources, and, consequently, abnormal gene expression and cleavage arrest during embryonic growth (Knijn *et al.*, 2002).

Thus, the environment in which oocytes develop is crucial because this greatly influences their ability to contribute to the creation of viable offspring. Success rates of IVM vary widely

among species. Maturation of bovine oocytes *in vitro*, for instance, is a well-established technique (Keskinetepe and Brackett, 1996; Rose-Hellekant *et al.*, 1998), but although IVM of non-human primate oocytes is feasible, success rates in terms of embryo production following IVF have been low (Schramm and Bavister, 1994, 1996a; Zheng *et al.*, 2001a, 2001b, 2002, 2003).

Aging effects

In addition to the problems that *in vitro* culture conditions may present for oocyte maturation, one must also consider age as a factor in reproductive competence. As the female primate ages, the ovary undergoes senescence culminating at menopause when the ovary has exhausted its reserve of viable follicles and oocytes (Ginsberg, 1991; Faddy and Gosden, 1996). The phenomenon of reservoir depletion and associated hormonal changes has been documented in several mammalian species, including humans and some non-human primates (Walker, 1995; Gilardi *et al.*, 1997; Miller *et al.*, 1999; Shideler *et al.*, 2001). Certainly, oocytes from aged females display increased incidences of chromosomal aberrations and mitochondrial defects (Liu and Keefe, 2002; Munne *et al.*, 2002). This may be due to prolonged storage (decades in apes and humans) in the ovary for the oocytes last to be recruited for ovulation (Eichenlaub-Ritter *et al.*, 2003).

In animals, the problem of aging effects on reproductive capabilities is perhaps most relevant in terms of captive breeding. Most animals living in their natural environment likely do not survive to reach “old age”. Sadly, due to deteriorating habitats, many higher primates suffer from an increased dependence on captive breeding and husbandry in order to survive. Implementation of effective SSPs is especially crucial for these captive populations and depends

partly on the development of viable ART options. Unfortunately, many zookeepers are hesitant to put into practice technologies that they may consider invasive and/or not proven successful. Therefore, it is imperative to develop technologies that are reliable and appropriate for each species and its situation when IVF or similar advanced ARTs are involved. This goal is dependent, in part, on the investigation of factors affecting proper oocyte maturation. Acceptance of such “artificial” approaches by the zoological community will certainly be helped, and may in fact depend upon, demonstrated successes in suitable model species. For example, the production of offspring by ART in rhesus monkeys (Bavister *et al.*, 1984) led to efforts to apply this approach to endangered lion-tailed macaques (Cranfield *et al.*, 1989, 1990).

Applications of IVM technology to various species are manifold and include: salvaging oocytes from genetically valuable individuals or from aging or moribund individuals, and the ability to harvest a useful number of oocytes per female. Research aimed at improving the quality of oocytes matured *in vitro* is critical and should include detection of various cytoskeletal and cytogenetic anomalies associated with IVM and aging. The correlation of such aberrations with developmental failure in IVM non-human primate oocytes may lead to viable strategies for supporting nuclear maturation. Using the rhesus macaque (*Macaca mulatta*) as a model, it is the aim of my research to contribute to this area as described below.

Aim 1: Ovarian senescence. Initial studies on reproductive competence in the macaque model examined morphological changes in the ovary associated with aging to establish a baseline by which to define the onset of senescence. Ovaries obtained from females over a wide age range (~1 – 25 years old), which included the entire reproductive spectrum, i.e., prepubescent to presumptively menopausal, were examined for ovarian follicle populations using basic

histological staining protocols. Based on earlier observations in the rhesus macaque, the reproductive spectrum approximates ages 4 - 25 years old (Gilardi *et al.*, 1997; Shideler *et al.*, 2001). Ovary samples from the various females were divided into several age groups in order to assess age-related trends in ovarian morphology.

The following aims relied on the availability of macaque oocytes. Rhesus macaque oocytes are a precious commodity and because the University of New Orleans does not have a primate research facility, I had to rely on collaborative efforts with such institutions. Therefore, partnerships between the University of New Orleans and the Tulane National Primate Research Center and the Caribbean Primate Research Center in Sabana Seca, Puerto Rico were established.

Aim 2: Frequency of aberrations in meiotic spindle structure in rhesus macaque. Metaphase spindle anomalies in oocytes were examined and compared among *in vivo* (IVO) and IVM oocytes and across age groups. Past studies in mice have demonstrated a correlation between faulty spindle formation and age (Hunt *et al.*, 1995) and *in vitro* culture of oocytes (Roberts *et al.*, 2005). In order to determine whether culture conditions and/or age influence spindle morphology within the rhesus macaque oocyte, a portion of mature oocytes from a given cohort were allocated to a staining protocol that defines the architecture of the meiotic spindle and chromatin formation using confocal microscopy. This was done using a monoclonal mouse antibody to α -tubulin to visualize microtubules, phalloidin (which binds actin) to visualize microfilaments, and a nuclear stain to visualize chromatin. Any disruptions or aberrations of the

cytoskeletal structure would certainly create conditions for problems, possibly lethal ones, to occur upon fertilization and subsequent development.

Aim 3: Frequency of aneuploidy in macaque gametes. Several factors relating to reproductive health and gamete competence must be considered when implementing programs for propagation of genetically valuable primates via natural breeding or ARTs. Relevant factors include ascertaining gamete chromosome normality of individuals. Cytogenetic aberrations in a portion of mature oocytes from a given cohort were examined and compared among IVO and IVM oocytes and across age groups. Fixation of macaque oocytes arrested at metaphase of meiosis II were performed following a modified air-drying technique originally developed by Kamiguchi *et al.* (1993). The macaque karyotype is composed of 42 chromosomes (including X and Y sex chromosomes) and has been previously described by Pearson *et al.* (1979) using information regarding chromosomal locations of functional genes, as well as the close resemblance of chromosomal banding patterns between rhesus monkeys and humans. Examination of aneuploidy rates among oocytes that have been matured and/or originate from various conditions (IVO vs. IVM, young vs. old females) will allow researchers to optimize conditions for oocyte maturation and, consequently, normal embryo development.

Accomplishment of the above stated aims will work in conjunction to broaden our base of knowledge regarding non-human primate oocyte maturation. Defining the onset of reproductive senescence in this species provides a baseline for assessing decreases in reproductive competence. Using this information, we can then investigate possible associations between karyotypic normalcy and cytoskeletal morphology with the onset of reproductive

senescence. Application of knowledge gleaned from these studies may help improve the current low developmental competence rates exhibited by primate oocytes that have undergone IVM prior to IVF.

Ideally, developmental competence of cohort oocytes would ideally be examined in conjunction with the above stated aims. A protocol for IVF in the rhesus macaque has already been established (Bavister *et al.*, 1984). Although it was my original intention to examine developmental competence of IVM oocytes, it was not logistically possible for reasons described later (see Chapter 3). However, it was possible for me to collect and examine semen samples to be used for this purpose later. It is of obvious importance that viable, quality sperm samples be available for IVF, hence an emphasis on cryopreservation of spermatozoa. Therefore, a fourth aim is indirectly relevant to the primary aim of improving oocyte quality:

Aim 4: Cryopreservation of rhesus spermatozoa. Semen cryopreservation is crucial for gene banking of genetically valuable animals and offers convenience for IVF experiments.

Cryobanking of spermatozoa can also reduce the frequency of ejaculation collections, reducing stress on the animals and making more efficient use of each semen sample collected. Several techniques have been described for cryopreserving spermatozoa of various non-human primate species (Kraemer and Vera Cruz, 1969; Lawrence *et al.*, 1976; Mahone and Dukelow, 1978; Tollner *et al.*, 1990; Sankai *et al.*, 1994). The purpose of this aim was to evaluate the effectiveness of several published primate sperm cryopreservation protocols including techniques that are not macaque-specific, to determine the most efficient one for the rhesus macaque. Successful sperm preservation protocols would contribute to the well-being of semen donors because the number of collections required for ART procedures could be reduced.

Extended ejaculates could be used for several experiments, eliminating possible male effects on the outcome of IVF procedures. The protocol found to be best for preserving rhesus macaque spermatozoa could then be used in IVF experiments aimed at assessing the developmental competence of IVM oocytes.

Chapter 2

Ovarian senescence in the rhesus macaque

Introduction

The primordial follicle population within an ovary represents the reservoir of potentially viable oocytes available for recruitment into the ovulatory pool. This population experiences huge decreases during the postnatal lifespan, partly due to follicular recruitment processes but mostly due to follicular atresia within the ovary. As the female ages, the ovary undergoes senescence, culminating in menopause when the ovary has exhausted its reserve of viable follicles and oocytes (Ginsberg, 1991; Faddy and Gosden, 1996). At this time, ovulatory activity ceases and estrogen levels will have decreased. In humans, women approaching or undergoing the menopausal transition or ‘climacteric’ may experience difficulty in conceiving and often resort to assisted reproductive techniques to achieve fertilization. In most of these cases, the woman will undergo an ovarian stimulation regimen to maximize follicle recruitment for IVF procedures. However, by this point, the ovaries may have lost the capacity to respond to such treatment due to diminished ovarian reserve and oocyte quality (Volarcik *et al.*, 1998; Hansen *et al.*, 2003). In non-human primates also, females that survive past their reproductive prime may experience these phenomena. This possibility holds many implications for breeding programs involving primate species whose natural populations may be threatened. Individuals housed in captive situations are likely to survive to old age and, therefore, the factor of age must be considered when constructing Species Survival Plans.

The development of a non-human primate model to investigate the etiology and occurrence of ovarian senescence is important. Female rhesus macaques are reproductively

active over a span of ~20 years (from 5 to 25 years of age) and undergo pathological and hormonal changes similar to those of the human female as she approaches the end of her reproductive lifespan (Walker, 1995; Gilardi *et al.*, 1997; Shideler *et al.*, 2001). These changes include a decrease in estrogen levels, the cessation of ovulatory events and an increase in circulating follicle stimulation hormone (FSH) levels (Walker, 1995). Early observations on total oocyte numbers within the rhesus ovary by Green and Zuckerman (1951) did not find a significant decrease with age. However, these studies utilized a small number of individuals (n = 12) from the beginning of the reproductive spectrum (aged 3-9 years) with ages being estimated by dentition and body weight. It would be expected that, at these earlier reproductive ages, major decreases in oocyte numbers would not be evident. The same investigators later added observations on total oocyte numbers in the rhesus ovary (Green and Zuckerman, 1954) and, based solely on the assumption that heavier individuals were older, determined that oocyte numbers did decrease with age. Recent studies in pigtailed macaques (*Macaca nemestrina*) demonstrate some of the pathological characteristics of approaching menopause (i.e., a significant decrease in primordial follicle numbers), although older females (aged 15-25 years) were not examined (Miller *et al.*, 1997, 1999). It may be concluded that aging studies utilizing older, peri- to post-menopausal monkeys are sparse.

In view of the limited number of studies on older non-human primates and the variation in reported menopausal details, the current project was conducted to examine ovarian senescence in the rhesus macaque across several age groups, from pre-pubertal to presumptively post-menopausal females (ages ~1–25 years), thereby covering the entire reproductive lifespan of this species. Results from histological examination provide evidence of follicular depletion over time, with a marked reduction at later ages.

Materials and methods

Female rhesus macaques of various ages (0.93-25.44 years) were systematically euthanized for medical reasons over a 5 year period at the Tulane National Primate Research Center (Covington, LA, USA). Ovaries were collected and preserved for later examination. These samples were divided into several age groups (<5 years, n = 12; 5-10 years, n = 14; 10-15 years, n = 14, 15-20 years, n = 11; >20 years, n = 13) in order to examine possible age-related trends in ovarian morphology. Serum samples from each euthanized female were also obtained at the time of necropsy and stored frozen for subsequent analysis of hormone profiles.

Histology

Ovaries were bisected and preserved in 10% formalin. Fixation was followed by a water rinse and subsequent storage in 70% ethanol. The tissues were subsequently embedded in paraffin wax (Fisher Scientific, Pittsburgh, PA, USA) following immersion in a graded series of alcohols (70-100%) and limonene-based clearing agent xylene (Fisher Scientific). Embedded tissue was sectioned (5µm) using a rotary microtome. Representative sections were adhered to microscope slides coated with gelatin (Sta-on; Surgipath, Richmond, IL, USA) and dried at 56°C for 24h.

Slides were stained using Harris' haematoxylin and eosin (HHE) to visualize general cellular structure throughout the tissue (Wheater *et al.*, 1987). Briefly, slides were exposed to the clearing agent Xylene and then rehydrated in a graded series of ethanols (100%, 100%, 100%, 95%, 80%). Following a bath in distilled water, slides were incubated in Harris' haematoxylin as the primary staining agent. The samples were then rinsed through a running tap water bath and Clarifier 1 (Richard-Allan Scientific, Kalamazoo, MI, USA) to remove excess

haematoxylin. Following another water bath, a strong basic solution of 0.3% ammonia water was used to shift the color of the haematoxylin in the sections to blue. This step was followed by another water bath, incubation in the eosin counterstain and subsequent dehydration in a graded series of ethanol (80%, 95%, 95%, 95%, 100%, 100%, 100%), and immersion in xylene, then glass coverslips were mounted on the slides with Cytoseal-60 (Stephens Scientific, USA).

Ovarian follicles were examined using a Nikon microscope (Nikon Instruments, Inc., USA). Follicles were counted and classified as primordial, primary or antral if they contained an oocyte in which the nucleus could be clearly defined. One ovary per individual was used based on previous finding by Miller *et al.* (1999) that symmetry of follicle numbers exists between ovaries in macaques. Because the aim of the study was to examine ovarian senescence occurring over female age, a representative single section was taken through the middle of each ovary and follicles were counted over the entire area. Although this approach precludes consideration of any possible heterogeneous distribution within the ovary, it was impractical to section the entire ovary of each animal. But as the size of each age group was sufficiently large and the sampling method was kept consistent throughout the study, it was considered that this method would provide an accurate reflection of any morphological changes.

Characterization of follicles as primordial, primary or antral was based on the criteria previously described by Bloom and Fawcett (1970). Primordial follicles contain oocytes with a large nucleus and pale dispersed chromatin (Figure 2.1 A). These oocytes are surrounded by a single layer of flattened granulosa or follicular cells. Primary follicles (Figure 2.1 B) possess either single or multiple layers of granulosa cells that are cuboidal or low columnar. The zona pellucida forms during the primary follicle stage and can be seen as a deeply staining layer between the oocyte and surrounding granulosa cells. Antral follicles (Figure 2.1 C) contain a

large fluid-filled space or antrum. At the antral stage, follicular cells continue to proliferate; however, the oocyte itself has already reached its full size.



Figure 2.1A-C. Various stages of ovarian follicle development. Primordial follicles (**A**) are characterized by a flattened granulosa cell border (arrows). Bar represents 25 μm . The primary follicle (**B**) exhibits several layers of granulosa cells and a zona pellucida surrounding the oocyte (arrow indicates germinal vesicle nucleus of the oocyte). Bar represents 25 μm . The antral follicle (**C**) contains a well-developed antrum (“a”) containing follicular fluid; arrow indicates oocyte within the follicle surrounded by modified granulosa (cumulus) cells. Bar represents 200 μm .

Photographs of ovarian sections were obtained using a Photometrics Coolsnap *cf* digital camera (Roper Scientific, USA) with a Nikon microscope equipped with Metamorph Imaging System software (Universal Imaging Corp., Downingtown, PA, USA).

Radioimmunoassay

Radioimmunoassay of sex hormones was conducted using Coat-A-Count kits purchased from Diagnostic Products Corporation (USA). These are no-extraction, solid phase radioimmunoassay designed for measurement of specific hormones in serum. Intra-assay and inter-assay precision averaged 5.3% and 5.7%, respectively.

Serum FSH concentrations were determined by radioimmunoassay in all samples at the completion of the study using protocols and reagents specifically developed for macaques

obtained from the National Institute of Health (NIH) National Hormone and Peptide Program directed by A.F. Parlow (Harbor-UCLA Medical Center, USA). The intra-assay coefficient of variation for FSH was 6.1%.

Statistics

Data were analyzed by least squares analysis of variance using the JMP software (SAS Institute, 2000). Individual means were compared by orthogonal contrast (SAS Institute Statistics and Graphics Guide, 2000). For the determination of number of primordial, primary and antral follicles, as well as for the results of the hormone assays, the age and number of parturitions of each female were entered into the model as effects.

Results

Follicles within each ovary section were examined, assigned to one of the three categories (Figure 2.1 A-C) and counted. Most sections revealed obvious and distinct changes in follicle populations with increasing age (Figure 2.2). Analysis of the proportions of the three follicle classes revealed that while the percentage of antral follicles remained nearly unchanged, the percentage of primary and primordial follicles changed significantly with age (Figure 2.3, $P < 0.05$). Analysis of the total number of primordial, primary and antral follicles within each section likewise showed a significant decrease with increasing age of the female (Figure 2.4 A-C).

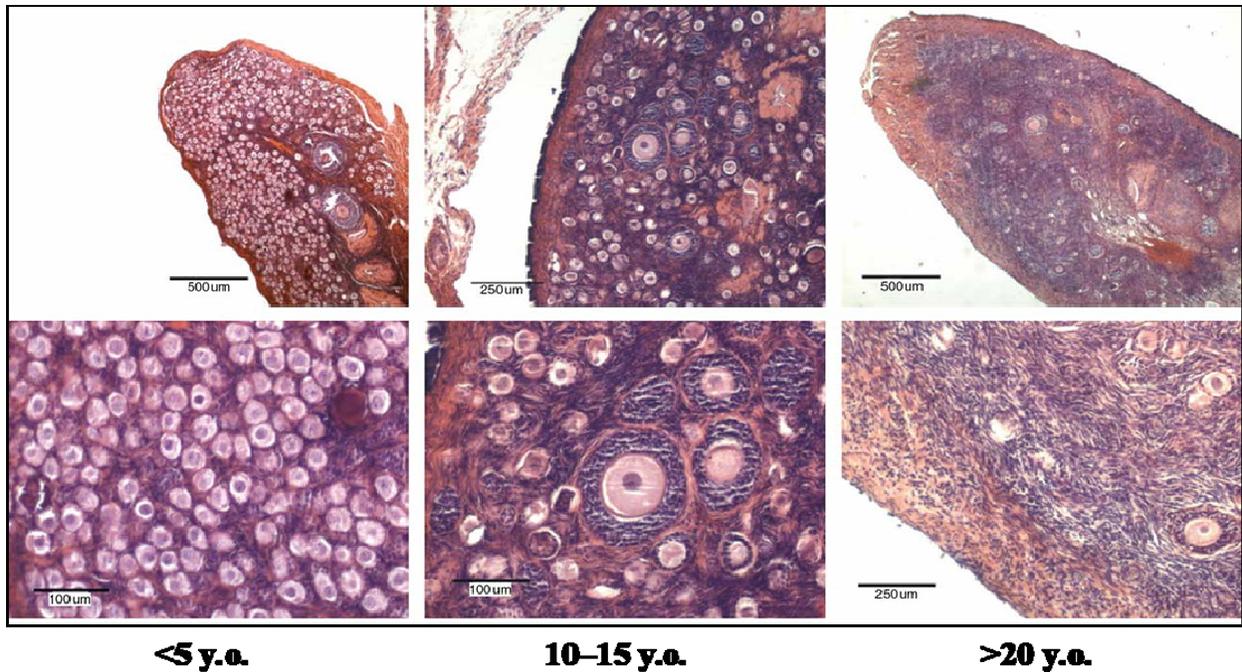


Figure 2.2. Ovarian sections illustrating changing follicle populations with increasing age.

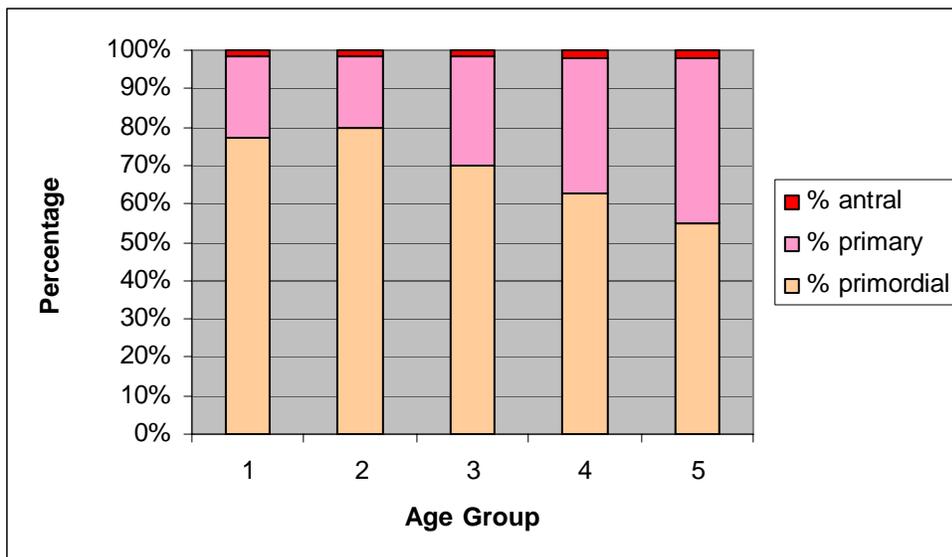


Figure 2.3. Effects of age on the proportion of primordial, primary and antral follicles. Age group 1, <5 years; group 2, 5-10 years; group 3, 10-15 years; group 4, 15-20 years; group 5, >20 years.

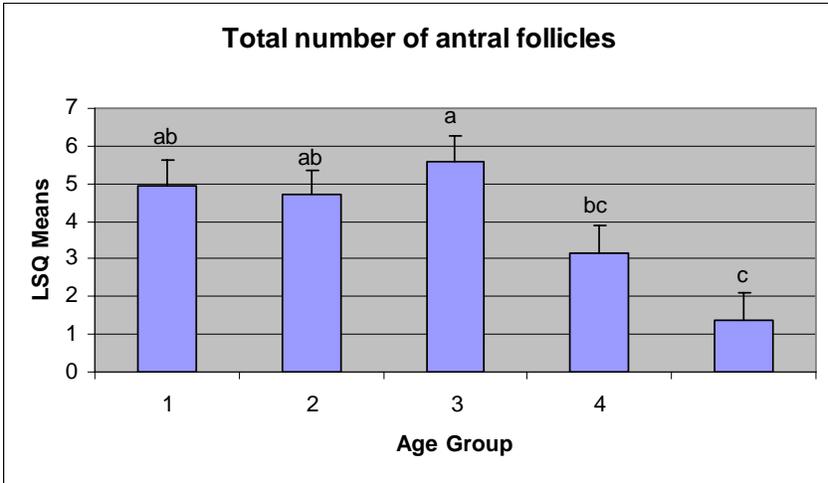
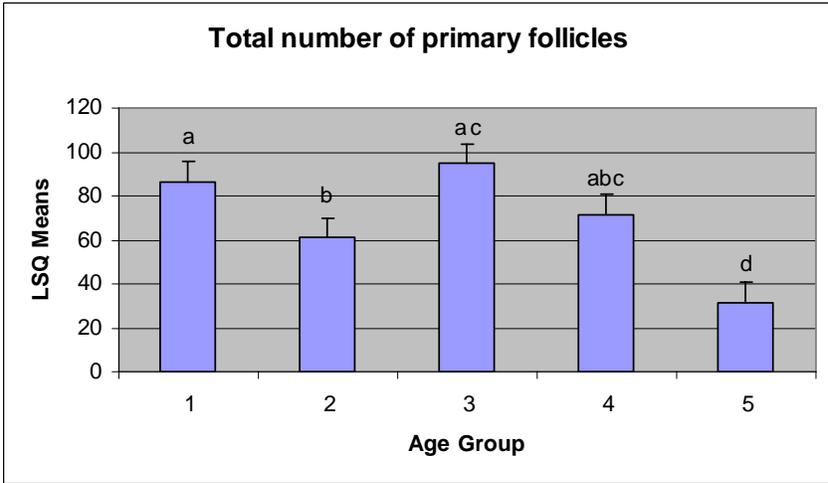
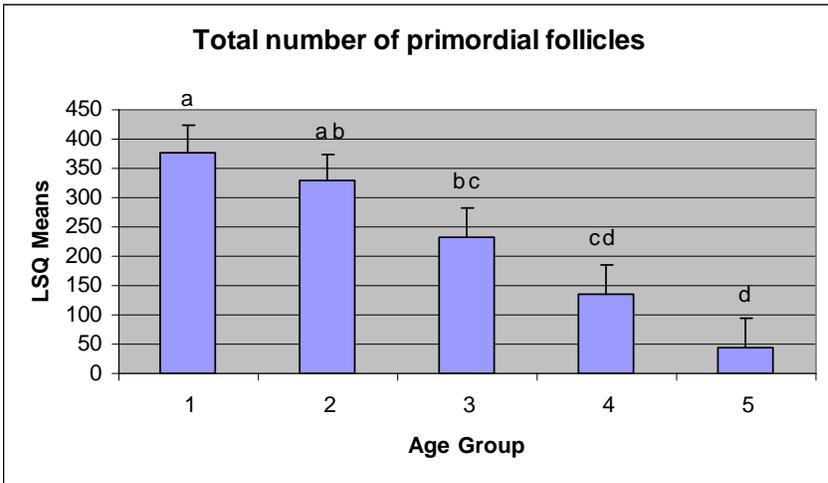


Figure 2.4 A-C. Follicle counts across age groups. Age group 1, <5 years; group 2, 5-10 years; group 3, 10-15 years; group 4, 15-20 years; group 5, >20 years. Different letters above error bars indicate significant difference in follicle number among age groups ($P < 0.05$).

Females undergoing the peri-menopausal and menopausal transition (age group 20-25 years) provided the most obvious visual evidence of ovarian reserve attrition, exhibiting scattered and atretic follicles, only occasional primordial follicles and reduced amounts of stromal tissue. Statistical analysis also demonstrated that the number of parturitions of each female had a significant effect on the proportion of primordial and primary follicles (Figure 5, $P < 0.01$) that was independent of age, with a decrease in the percentage of primordial follicles and a concomitant increase in mean percentage of primary follicles.

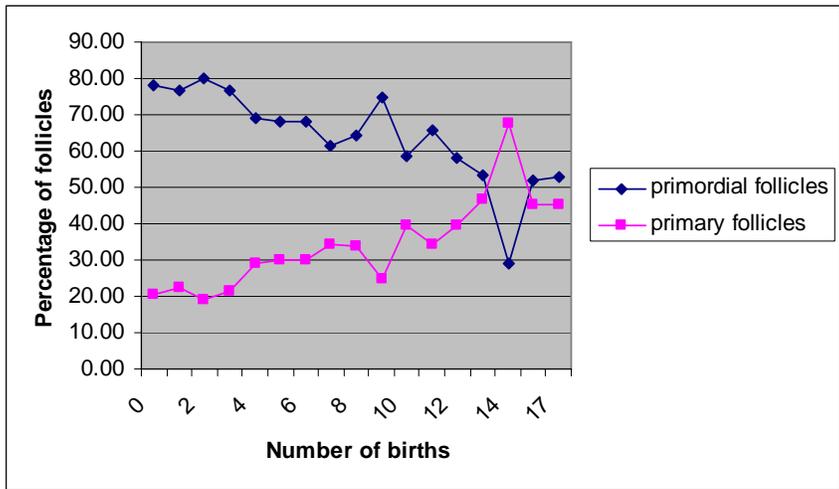


Figure 2.5. Effect of the number of parturitions on the proportion of primordial and primary follicles.

Analyses of blood samples taken at the time of euthanasia for concentration of estrogen, progesterone and FSH did not show any difference among females of various ages.

Discussion

This study describes ovarian follicle morphology and depletion in rhesus macaques from pre-pubescence until menopausal stages. It expands the observations of Gilardi *et al.* (1997) in describing ovarian senescence in aging laboratory rhesus macaques by including individuals from the entire reproductive lifespan. Samples from female macaques approaching or undergoing the menopausal transition (age group 20-25 years) provided evidence of ovarian attrition, having scattered and atretic follicles, only occasional primordial follicles and reduced amounts of stromal tissue (Figure 2.2).

Ovaries of young female rhesus monkeys contained large populations of primordial follicles, the numbers of which decreased significantly with age (Figure 2.4). Decreases in primordial follicle populations can be, in part, attributed to follicle recruitment during the follicular phase of the menstrual cycle. The factors determining which follicles are recruited to grow and develop during a reproductive cycle remain undefined, but growth factors and gonadotropins are believed to be necessary to sequentially activate initial follicular phase growth phases. The majority of the follicle population decrease, however, is due to natural attrition. Follicular atresia or degeneration is a prominent feature in the life of the mammalian ovary, beginning *in utero*, occurring primarily at birth and before puberty (Baker, 1963; Forabosco *et al.*, 1991; Faddy, 2000) and continuing at a slower rate throughout reproductive life. It is intriguing that Johnson *et al.* (2004) described the persistence of germline stem cells in the mouse ovary, which appears to challenge the long-held view that females have finite gamete reserves. In their mouse model, germline stem cells present within the postnatal ovary appear to continue with oogenesis, thereby slowing down the apparent rate of follicular attrition. This

controversial observation may have significant implications for mammalian reproduction, although it remains to be determined whether this phenomenon also occurs in other species.

The present study showed that the rhesus macaque ovary exhibits an age-related pattern of follicle depletion similar to that described in the human ovary (Richardson *et al.*, 1987; Faddy *et al.*, 1992; Faddy, 2000). As the rhesus female approaches the third decade of life, morphological characteristics of the menopausal transition or climacteric become evident. These characteristics include loss of stromal tissue and extremely low populations of primordial, primary and antral follicles, many of which have become atretic (Figure 2.2).

Although total numbers of primary follicles within representative sections examined decreased with age, the percentage of the total follicle population represented by this stage increased over time (Figure 2.3). The decrease in number of primary follicles reflects the degenerative processes occurring within the ovary. The increase in population percentage of the primary follicles may be explained through the natural process of primordial follicle recruitment during the follicular phase, in which selected follicles are initiated into growth and begin to proliferate the granulosa cell layers surrounding the oocyte. This process is under the control of growth hormones and periodically results in a fraction of the primordial follicles being converted into primary follicles (Webb *et al.*, 2003). Further growth is gonadotropin-dependent (Macklon and Fauser, 1999) and many of the newly formed primary follicles will cease development, possibly due to a lack of FSH receptors on the surrounding follicular cells, and so become arrested at the primary stage or become atretic. When number of births was considered, there was a significant increase in the percentage of primary follicles with increasing numbers of parturitions. The cause of this intriguing observation is not clear, but presumably a female who gives birth to a larger number of offspring is pregnant for much of her reproductive lifespan.

During pregnancy, the menstrual cycle ceases as a result of the continued production of progesterone from the corpus luteum and later from the placenta, which blocks the pituitary from producing FSH (Pohl *et al.*, 1982). Consequently, primary follicle recruitment and growth is halted, while recruitment and loss of primordial follicles would continue throughout life.

During the menopausal transition of rhesus females, there is a marked variability in levels of reproductive hormones (Gilardi *et al.*, 1997; Shideler *et al.*, 2001). Reproductive hormone variability may be a result of follicular depletion as proposed for the human female (O'Connor *et al.*, 2001). As the female ages, there is a shift and decrease in overall follicular hormone signaling, partly due to decreases in total numbers of follicles and possibly also due to the quality of remaining oocytes. In this study, because there were no available comparative data for the female macaques nor information regarding stage of menstrual cycle at the time of their deaths, the single time-point measurements did not provide useful information and, not surprisingly, no age-related differences were observed in sex hormone or gonadotropin levels. Further studies may include an examination of long-term changes in hormone profiles associated with the end of reproductive life through controlled sequential blood samples taken over prolonged periods of time before and after menopause.

In conclusion, the current study demonstrates striking similarities between rhesus monkeys and humans in their changing follicular population distributions related to reproductive senescence. Future studies with the rhesus macaque model could be useful for understanding the mechanisms underlying reproductive aging so that assisted reproductive technologies can be devised to counter this phenomenon. This information will be especially important for the application of ART to females that are nearing the end of their reproductive lifespan.

Acknowledgements

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Permission from the coauthors for use of this publication can be found in Appendix 1.

Chapter 3

Establishment of an embryology research laboratory at the Caribbean Primate Research Center

Introduction

As mentioned earlier in Chapter 1, establishing collaborative relationships with a primate research facility is crucial in order to conduct reproductive research in non-human primate models. Much of the research effort at the eight federal National Primate Research Centers in the United States is committed to studies on disease and especially virology research (primarily involving AIDS). Because of the huge demand for monkeys dedicated to these studies, animals available for other uses such as reproductive investigations are limited in number, difficult to obtain and very costly. As a result, to obtain sufficient materials for my research it was necessary to explore other options. One attractive possibility was to work with the Sabana Seca Field Station of the Caribbean Primate Research Center (CPRC) in Puerto Rico. During late Summer of 2005, I arrived at that facility to study assisted reproduction in the rhesus macaque and to complete my dissertation studies on the analysis of oocyte quality in the macaque. However, the CPRC's research mission for the past 60 years has primarily focused on behavioral investigations. Earlier efforts at establishing an embryology laboratory at the CPRC had not been successful and the CPRC was eager to have this program materialize.

The CPRC animal colony was founded in 1938 with the importation of rhesus macaques from India and has remained a closed colony of this subspecies (for a full description of the history and facilities of the CPRC see Appendix 1), whereas the national primate centers on the U.S. mainland (which were in many cases partly seeded by animals from the CPRC) include imported Chinese as well as Indian and Chinese-Indian hybrid rhesus macaques. Significant

differences have been documented among Indian-origin, Chinese-origin and hybrid rhesus macaques at the genetic, physiologic, morphometric and behavioral levels (Champoux *et al.*, 1997; Clarke and O'Neil, 1999; Viray *et al.*, 2001; Trichel *et al.*, 2002; Smith, 2005; Ferguson *et al.*, 2007) which may include response to exogenous gonadotropins (Nichols and Bavister, personal observations). These differences can influence the results of experiments.

Consequently, it is often a concern to determine the origin of individual macaques allocated for certain research projects. Because the macaques at the CPRC are of exclusively Indian origin, these animals offer a unique opportunity for biomedical studies. The CPRC has a large population of animals available for research which are not constrained by large AIDS programs like the mainland primate centers. Thus, the situation at CPRC was ripe with opportunity for our laboratory's research.

Experimental research in reproduction at the CPRC.

Initial work beginning in Fall of 2005 focused on the implementation of previously developed hormonal stimulation protocols for rhesus macaques in order to establish basic parameters, e.g., the average number of oocytes collected per stimulation cycle, so that an experimental plan could be instituted. While other primate research centers have reported on numbers of oocytes per female per stimulation cycle and the number of responsive cycles obtained (Zelinski-Wooten *et al.*, 1994; VandeVoort and Tarantal, 2001; Stouffer and Zelinski-Wooten, 2004), these baselines had not been previously examined at the CPRC. With the recognized disparities between rhesus macaques of different origins in mind, there was an element of uncertainty as I suspected that the CPRC animals might not respond identically to hormonal stimulation as macaques in the other national primate research centers.

A study group of 20 females was chosen based on their health, history of successful births and age. Geriatric females (age 17 and over) were included in the group in order to investigate possible age-related trends. Rhesus macaques are seasonal breeders whose reproductive season spans from September through early May. The average menstrual cycle lasts 28 days in this species (Jenkin *et al.*, 1980; Danforth *et al.*, 1989), sharing similar timing and hormonal patterns with the human menstrual cycle. The macaque is monovular, i.e., normally ovulating only one oocyte per cycle. In order to override the selection of a single dominant follicle, exogenous recombinant follicle stimulation hormone (r-FSH) is administered to stimulate the development of multiple large antral follicles with enclosed oocytes that have the potential for successful fertilization (Stouffer and Zelinski-Wooten, 2004). An injection of the LH-like hormone recombinant human chorionic gonadotropin (r-hCG) is administered to mimic the luteinizing hormone (LH) surge of the menstrual cycle, which acts to release oocytes from meiotic arrest so that they reach metaphase II and become capable of fertilization (Figure 3.1).

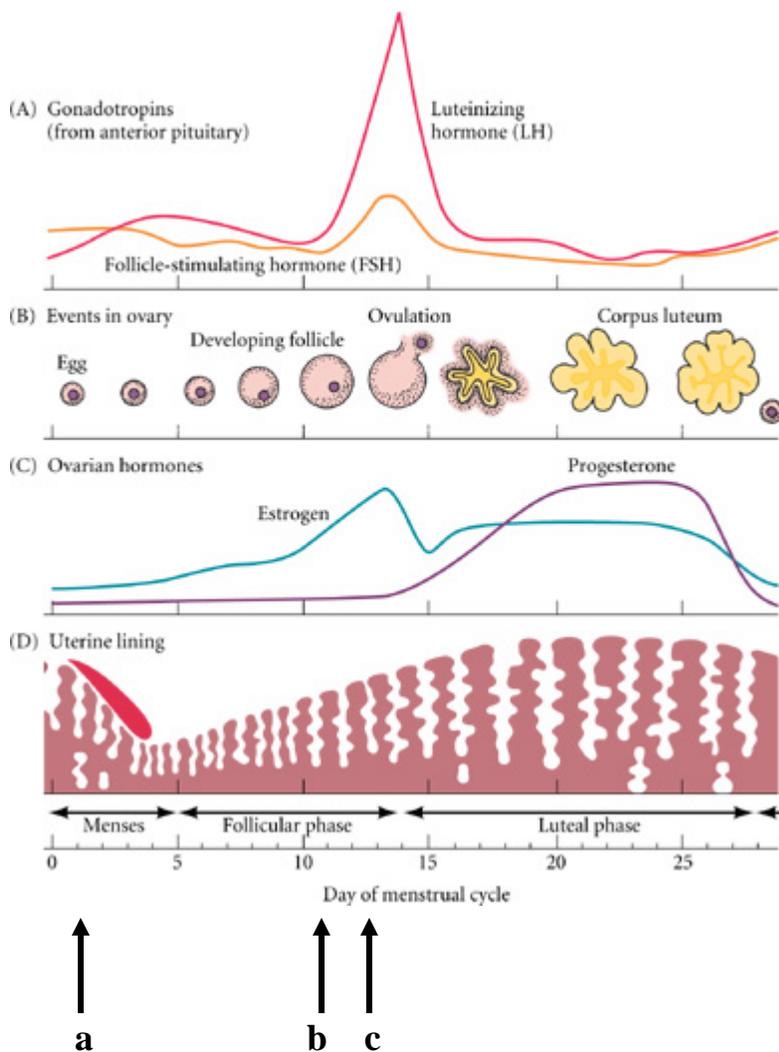


Figure 3.1. The menstrual cycle pattern of both humans and rhesus macaques. Pituitary (A) and ovarian hormones (C) act to coordinate ovarian events (B) and uterine conditioning (D). The oocyte matures within the follicle at the end of the follicular phase while the uterine lining is undergoing proliferation and preparation for blastocyst implantation. The mature oocyte is normally ovulated around day 14. If fertilization and blastocyst implantation do not occur, the uterine wall begins to break down, leading to menstruation. Administration of exogenous r-FSH (**a**) begins on Day 1, 2 or 3 of the menstrual cycle and continues for 8-10 days. A bolus of r-hCG (**b**) is injected towards the end of the follicular phase with aspiration (**c**) of stimulated ovarian follicles 27-33 hours later. (Menstrual cycle graph obtained online at <http://8e.devbio.com>)

Females in the study were monitored on a daily basis for onset of menses during the breeding season. Recombinant follicle stimulating hormone (Gonal-F, Serono Laboratories, Rockland, MA, USA or Follitropin Beta, Organon Inc., West Orange, NJ, USA) was administered twice daily (~37.5IU/injection) beginning on days 1-3 of the menstrual cycle (day 1 = first day of menstruation). Injections were performed with the aid of veterinary technicians. The standard hormonal stimulation protocol in the rhesus macaque established by the Bavister laboratory (Schramm and Bavister, 1996b) consisted of 8 days of r-FSH alone, followed by an injection (1000 IU) of r-hCG (Zelinski-Wooten *et al.*, 1997) to induce maturation of oocytes. Laparoscopic aspiration of the ovarian follicular contents was performed between 27-33 hours post-r-hCG (Wolf *et al.*, 1996). Females were ‘rested’ for one month between stimulation cycles. This protocol was followed during the Fall of 2005 with highly variable results in terms of numbers of oocytes collected per cycle and percentages of mature oocytes within a collected cohort. Initial cycles averaged 24.6 ± 10.7 (st. dev.) oocytes per collection (range 7 – 33 oocytes, n = 5 females: 2 geriatric, 3 young females), however, none of the retrieved oocytes were mature (reached metaphase II) at aspiration in any of these initial cycles. The time between the r-hCG injection and retrieval was increased from 27 hours to 30 hours and then to 33 hours in order to allow oocytes as much time as possible to mature *in vivo* without risking their loss due to ovulation. It was also suspected that the potency of our r-hCG (Ovidrel, Serono, Rockland, MA, USA) had attenuated. This hormone is a light- and time-sensitive liquid product, susceptible to fluctuations in temperature and with a short effective lifespan. According to the manufacturer, the product “must be stored refrigerated between 2 – 8 °C until expiration” and cannot be frozen. If stored at room temperature (up to 25°C), it “must be used within 30 days”. Ovidrel was originally shipped to the CPRC from New Orleans, Louisiana (via Texas!)

following the Hurricane Katrina disaster and it is likely that our supply underwent major temperature variations. Fresh Ovidrel was therefore obtained, retrievals were set at 33 hours post-r-hCG and the 2 remaining cycles during Fall 2005 showed improvement. Ten oocytes were collected from one female (a geriatric individual), of which 4 were mature upon aspiration, and another 36 oocytes were collected from a young individual (~9 years old), 20 of which were mature upon collection (40% and 56% *in vivo* matured (IVO), respectively). These results were promising.

When the Center reopened in early January 2006, observations on study group females and institution of hormonal stimulations were resumed. The 10 day stimulation protocol was continued. The first 10 cycles of 2006 averaged 22.4 ± 6.2 oocytes per cycle. *In vivo* maturation rates ranged from 0 to 50%. This was not acceptable. These animals were still not responding as expected, perhaps due to the origin of these particular rhesus macaques (purebred Indian rhesus). So, the stimulation protocol was adjusted again. The revised protocol consisted of 8 days of r-FSH alone (37.5 IU, 2x/day), two days of r-FSH (37.5 IU, 2x/day) *plus* administration of “mini” r-hCG injections (50IU each, 2x/day) to mimic the natural rise in LH at the end of the follicular phase. This was followed by the usual 1000 IU injection of r-hCG, with aspiration taking place, on average, 33 hours post-r-hCG injection. Five cycles were performed. It should be noted that during one of these cycles, there was a power outage at the facility and the animal could not undergo laparoscopy until much later post-r-hCG injection, i.e., over 36 hours later. Not surprisingly, this female had ovulated, although there were still some large unruptured follicles that the veterinarian was able to aspirate. Seven oocytes were collected from this female, bringing the average number of oocytes collected from these 5 cycles down to 16.8 ± 6.6 from 19.3 ± 4.3 oocytes per cycle for this group of females. The percentage of IVO oocytes during

the first 5 trials of this extended stimulation protocol ranged from 26% up to 86% (26, 48, 54, 75, and 87%). These results were an improvement in overall oocyte maturation and this extended protocol was then adopted as the new standard for the CPRC reproductive program. The reason for this improvement was suspected to be the increase in total r-FSH and in days of the stimulation cycle. It is well documented that FSH induces the granulosa cells of the follicle to synthesize LH receptors and acquire the ability to respond to its stimulatory effects (Erickson *et al.*, 1979; Erickson, 1996). The increase of exogenous FSH dosage and length of stimulation cycle may have finally provided enough time for the granulosa cells to develop LH receptors and react to r-hCG administration in this particular population of macaques.

By mid-May 2006, the females entered the off-season and although a few trial stimulations were attempted, they were quickly halted because they yielded very poor responses from females who normally responded well. Females began normal menses again in September. Preliminary results showed that it was important to recognize the highly variable responses to gonadotropin stimulation in the CPRC rhesus macaques, and take this into account in my experimental design. According to the CPRC Institutional Animal Care and Use Committee, gonadotropin stimulations are limited to a maximum of 4 cycles. However, there was a variable response to successive stimulations. Some individuals continue to respond well, others become refractory (VandeVoort and Tarantal, 2001). Females that responded poorly to exogenous hormonal stimulation from their first cycles or that did not respond well to successive stimulation cycles were dropped from study. Additional females meeting the original requirements were added to the study group. The program for Fall of 2006 began with 20 females, some of which were new to the study. Females from this group would contribute most to the completion of my research into the effect of IVM and age on oocyte karyotype and spindle morphology (see

Chapter 4). Stimulation protocols were executed in one of two ways – either the new full stimulation protocol developed during Spring 2006 to produce IVO oocytes or the same stimulation regimen minus any r-hCG exposure, i.e., an r-FSH-only cycle, to provide immature oocytes that should undergo IVM after aspiration.

Total numbers of oocytes collected per aspiration during Fall 2006 and January 2007 averaged 16 ± 7 over 24 cycles (including full stimulation and FSH-only cycles). It should be noted that the majority of females contributing to this set were undergoing stimulation for a 3rd and 4th time, providing a possible explanation for the relatively low collection numbers when compared to other studies (Zelinski-Wooten *et al.*, 1994). Of the 24 cycles performed, 12 were full stimulation cycles (r-FSH and r-hCG) with an average IVO rate of $60 \pm 13\%$. The immature oocytes collected during these full stimulation cycles underwent IVM at a rate of $74 \pm 14\%$. Oocytes originating from r-FSH-only cycles averaged an IVM rate of $81 \pm 9\%$. Each cohort from these collections was split into two fixation protocols for analysis.

There remains room for improvement in number of oocytes collected per cycle and rate of IVO. Other primate centers have reported average collections between 20-30 oocytes with IVO rates of approximately 76% (Schramm and Bavister, 1996b, Stouffer and Zelinski-Wooten, 2004). Ideally, stimulations at the CPRC will attain these standards. This may be possible through the exploration of different sources of r-FSH and r-hCG and further modifications of the stimulation protocol.

Chapter 4

Effects of *in vitro* maturation and age on oocyte metaphase spindle integrity and chromosome segregation

Introduction

In mammals, the term “oocyte maturation” is generally limited to refer to the progression of a germinal vesicle stage oocyte to the metaphase stage of meiosis II, an event that is more accurately called “nuclear maturation” (Figure 1.2). Nuclear maturation is a descriptive term incorporating germinal vesicle breakdown (*GVBD*) and extrusion of the first polar body and does not adequately cover accompanying events in the cytoplasm, so-called “cytoplasmic maturation.” The latter events are also essential for fertilization and support of embryo development but are very poorly understood, even in rodent models, and virtually nothing is known about them in primates. Because aspects of nuclear maturation are much more feasible to study in primate oocytes, this study was limited to examination of two key components of nuclear maturation: oocyte chromosome complement (karyotype) and integrity of the meiotic spindle. These components are functionally linked because incorrect spindle assembly or disruption during meiosis I will most likely result in anomalies of chromosome segregation (non-disjunction) that will likely perturb early development after fertilization. Moreover, nuclear maturation anomalies are more likely to occur as a result of maturation of oocytes *in vitro*, which is an important component of Assisted Reproductive Technologies; and increasing age of females is known to be associated with decreasing oocyte competence in women undergoing ART in infertility clinics. Accordingly, this study examined oocyte karyotype and spindle integrity in rhesus monkey

oocytes, and attempted to correlate anomalies with *in vitro* oocyte maturation and with age of oocyte donors.

There are two arrest points during meiosis in the primate oocyte, namely at prophase I and metaphase II. Once it is signaled to resume meiosis, the prophase I oocyte undergoes GVBD, assembly of the first meiotic spindle and alignment of paired homologous chromosomes (each consisting of two sister chromatids) on the spindle equator prior to their timely segregation during anaphase I. The oocyte then undergoes the first meiotic division (resulting in extrusion of the first polar body), reassembly of the meiotic spindle and alignment of the chromosomes (consisting of a pair of sister chromatids) along the spindle equator, and meiosis arrests again at metaphase II. At this point, the primate oocyte is mature and receptive to fertilization.

Over 70 years ago, Pincus and Enzmann (1935) first demonstrated that immature oocytes could spontaneously undergo *in vitro* maturation (IVM) and this technique has been continuously improved since then. Together with *in vitro* fertilization (IVF) and cryopreservation, the ability to successfully perform oocyte IVM has great potential as a tool in the conservation of endangered species, in commercial production of embryos from domestic species, in assisted reproduction for infertile women, and for basic understanding of oocyte biology. Although normal offspring have been produced using IVM/IVF technology in humans (Trounson *et al.*, 1994; Barnes *et al.*, 1995; Russell *et al.*, 1997) and in monkeys (Schramm and Paprocki, 2000), IVM is far from being a reliable technique for the production of developmentally competent oocytes in primates, unlike in cattle (Hasler *et al.*, 1995). *In vitro* matured primate oocytes from unstimulated or from FSH-stimulated monkeys display reduced developmental competence compared with *in vivo* matured (IVO) primate oocytes (Schramm and Bavister, 1996a, 1996b; Gilchrist *et al.*, 1997; Zheng *et al.*, 2001a, 2001b, 2003) or IVM oocytes of other mammalian

species (mice, Schroeder and Eppig, 1984; Eppig and Schroeder, 1989; sheep, Morton *et al.*, 2005; cattle, Coleman *et al.*, 2007). Impaired developmental competence may be due to chromosomal aberrations, as commonly seen in human oocytes (Hassold and Hunt, 2001), resulting from suboptimal culture conditions and/or from excessive gonadotropin stimulation. Proper maturation requires faithful congression and division of chromosomes as well as other less well-understood changes comprising cytoplasmic maturation.

The meiotic spindle is highly dynamic and its structural integrity is fundamental to successful chromosome congression, segregation and cytoplasmic partitioning. This bipolar structure is composed of microtubules constructed from polymerized α - and β -tubulin dimers and various proteins such as dynein and kinesin (Barton and Goldstein, 1996) which guide movement of chromosomes during meiosis. Disturbances in spindle formation and function may create conditions for aneuploidy and/or maturation arrest through depolymerization of microtubules. The term aneuploidy denotes any deviation from the diploid number of chromosomes that is characteristic of a given species. In unfertilized oocytes, the term aneuploidy describes chromosomal aberrations in which one or more chromosome(s) may be missing (hypohaploidy) or extra chromosome copies may be present (hyperhaploidy). Embryos created from fertilization of aneuploid oocytes inherit these anomalies which are usually lethal to embryo development. In fact, chromosome abnormality is the most common recognized cause of fetal death in humans (Nicolaidis and Petersen, 1998). It is essential that meiotic spindles remain intact during handling of oocytes in assisted reproduction to retain faithful chromosome congression and segregation. However, spindle formation and chromosome segregation are particularly sensitive to the physical and chemical environment (Albertini 1992; Wu *et al.*, 1999; Sanfins *et al.*, 2003), emphasizing the importance of oocyte maturation conditions.

In humans, the frequency of oocyte and embryo aneuploidy increases significantly with age (Hassold and Chiu, 1985; Nakaoka *et al.*, 1998; Pellestor *et al.*, 2003). Although the underlying mechanism is not clear, the majority of these aneuploidies are believed to be a result of chromosome non-disjunction and meiotic errors initiated during meiosis I (Battaglia *et al.*, 1996; Angell, 1997). Initial meiotic arrest at the diplotene stage of prophase I occurs prenatally and can be suspended for decades in long-lived species such as humans and macaque monkeys. During this long period of time, damage accumulated in the oocyte or their companion somatic cells may be more severe than in short-lived species, e.g., mice (Eichenlaub-Ritter *et al.*, 2003), and could account for age-related fertility decreases observed in primates. Correspondingly, non-disjunction in mouse oocytes during reproductive aging is uncommon (Golbus, 1983; Sugawara and Mikamo, 1986; Sakurada *et al.*, 1996).

Development of a consistently successful IVM method for the production of developmentally competent primate oocytes has important implications for attempts to apply assisted reproductive technologies to conserve threatened or endangered non-human primates, especially long-lived captive individuals. These include the ability to salvage oocytes from genetically valuable females (relevant to species survival plans), including old and/or moribund individuals, and the ability to harvest a greater number of fertilizable oocytes per female. To date, a method for reliably maturing primate oocytes *in vitro* and producing oocytes that support successful embryo development at the same rate as IVO oocytes does not exist. If IVM is to be applied to conservation efforts, success rates must be improved. Efforts should include investigation into factors that may affect timely and accurate meiotic spindle formation and maintenance, and incidences of chromosomal aberrations.

This chapter describes research into the possible effects of IVM and age of female on oocyte quality in the rhesus macaque. The study was designed to test the null hypothesis that there are no differences between IVO and IVM primate oocytes in the frequencies of aneuploidy and abnormal meiotic spindle arrangements.

Materials and Methods

All animals were used with approval of the University of Puerto Rico's Caribbean Primate Research Center (CPRC) Institutional Animal Care and Use Committee. Ten females (7 young, 3 geriatric females) were allocated for this study. Females were considered reproductively geriatric if ≥ 17 yrs of age based on my previous observations on ovarian senescence in the rhesus macaque (Chapter 2). To reduce the effect of between-female variability, each female underwent both Regimen A and B stimulation cycles (see below). A rest period of at least one month was given between stimulation cycles. Every cohort of oocytes was randomly divided for analysis of spindle morphology and karyotype. All chemicals used in the study were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Oocyte collection

Regimen A

Reproductively active female macaques were monitored for regular menstrual cycles. When menses occurred, the females were stimulated with a recombinant human follicle stimulating hormone (r-FSH, Follitropin Beta, Organon Inc., West Orange, NJ, USA) (dosage = ~ 37.5 IU/2 x d for 10 days) through intramuscular injections beginning on day 1, 2 or 3 of the onset of menses. Ultrasound was performed on day 7 or 8 of the stimulation cycle to monitor

response of follicles to exogenous hormone. The last two days of r-FSH administration included small doses (50IU/2 x day) of recombinant human chorionic gonadotropin (r-hCG, Ovidrel, Serono Laboratories, Rockland, MA, USA) prior to one final injection of r-hCG (1000 IU) to induce oocyte maturation. Oocyte retrieval was performed laparoscopically approximately 33 hours after r-hCG injection and the follicular aspirates examined for the presence of oocytes. *In vivo* matured oocytes (oocytes at metaphase of meiosis II, as evidenced by extrusion of a polar body) from each cohort were randomly fixed for either spindle or karyotype analysis. Immature oocytes (germinal vesicle stage or germinal vesicle breakdown stage oocytes) from these cohorts were placed into IVM medium (mCMRL-1066; Zheng *et al.*, 2001a) that had been pre-equilibrated by incubation at 37°C, 5% CO₂ in air overnight and examined the following day for the presence of a polar body. Mature oocytes from this group were also randomly fixed for analysis and represented a subclass of IVM oocytes.

Regimen B

Regimen B followed the same protocol as Regimen A except that the females were given r-FSH only, i.e., 10 days of r-FSH (~37.5 IU/2 x d), 1 day of no injections and follicular aspiration was done on the 12th day of the stimulation cycle. The aspirated, immature oocytes were placed into IVM medium (mCMRL-1066) that had been pre-equilibrated at 37°C, 5% CO₂ in air overnight and examined the following day for the presence of a polar body. Mature oocytes from each cohort were randomly fixed for analysis as for Regimen A.

Oocyte Fixation

Chromosome Spreading

Mature oocytes were fixed for chromosome analysis using a gradual fixation technique previously described by Kamiguchi *et al.* (1993). Briefly, metaphase II oocytes were denuded of any remaining cumulus cells by rapid pipetting and placed into acid Tyrode's solution to dissolve the zona pellucida. Following zona pellucida removal, the oocyte was placed into hypotonic solution (0.9% sodium citrate:bovine calf serum, 3:1, 37°C) for 15 min. The expanded oocyte was incubated in fixative I (methanol:acetic acid:distilled water, 5:4:1) for 5 min. The oocyte was then placed onto a grease-free slide and a small amount of fixative II (methanol:acetic acid, 3:1) was dropped onto the oocyte prior to placing the slide into a Coplin jar filled with fixative II for 10 min. Following incubation in fixative II, the slide was placed into fixative III (methanol:acetic acid: distilled water, 3:3:1) for 1 min, then slowly removed and dried in warm moist air.

Each slide was initially examined with a 40X objective on a Nikon microscope to determine success of chromosome spreading. Samples in which the chromosomes appeared well-spread were air dried at room temperature prior to staining with Giemsa:Sorensen's buffer (2:21) for 2 min. Due to the peculiar morphology of primate oocyte chromosomes with highly condensed chromatin, it was not possible to obtain quality banding for individual chromosome identification. The diploid chromosome number for the rhesus macaque is 42, with 20 pairs of autosomes and two sex chromosomes (Stock and Hsu, 1973). Metaphase spreads were scored for incidences of aneuploidy under light microscopy by two investigators (myself and Dr. Hilla Azoulay, University of Puerto Rico, Rio Piedras), the latter scoring blindly.

Cytoskeletal Staining

Mature denuded oocytes from the same cohorts as their karyotyped counterparts were transferred soon after aspiration into a microtubule stabilizing fixative (2% paraformaldehyde, 0.1% Triton X-100, 1 μ M paclitaxel, 0.01% aprotinin, and 1 mM dithiothreitol in water) for 30 minutes. Following fixation, the oocytes were placed into a block solution (2% BSA, 2% non-fat powdered milk, 2% normal goat serum, 0.1 M glycine, and 0.01% Triton X-100 in PIPES buffer solution, pH 6.9) at 4°C overnight in order to reduce free aldehydes and prevent background interference with immunostains. Preserved samples were then washed extensively and incubated with a mouse anti α -tubulin monoclonal antibody (MP Biomedicals, Inc., Aurora, OH, USA) for 1 h at 37°C, washed, and then incubated with rhodamine-conjugated goat anti-mouse IgG (MP Biomedicals) for 1 h at 37°C. After washing, samples were stained for actin filaments with Phalloidin, Alexa-488 conjugate (Invitrogen/Molecular Probes, Eugene, OR, USA) for 1 h, washed again and incubated for 20-30 min in To-Pro3 (Invitrogen/Molecular Probes) to stain DNA. Staining of actin filaments was incorporated to facilitate distinction of the polar body and its contents. Finally, a glass coverslip was placed on each slide with ProLong® Gold antifade mounting medium (Invitrogen/Molecular Probes) to prevent photobleaching and the slide was placed in 4°C until imaged.

Stained samples were visualized using a confocal microscope (Zeiss Axiovert 100M LSM 510) and LSM 510 software (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). Fluorescent probes were excited with the following lasers: Alexa-488 – 488 laser; rhodamine – 543 laser; and To-Pro3 – 633 laser. Images were captured at 100x and z-stack images were also obtained to create 3-dimensional projections of each sample. Spindle images were scored for

incidences of abnormal meiotic spindle arrangements by two investigators (myself and Dr. Hilla Azoulay, University of Puerto Rico, Rio Piedras), the latter scoring blindly.

Statistical Analysis

Statistical analysis of rates of aneuploidy and spindle aberrations was performed using 2x2 G tests described in Sokal and Rohlf (1981) to determine significant differences. The G test incorporates an arcsine transformation of the percentage values so that the data conform to statistical assumption of normal distribution. The statistical formula used was

$$t_s = \frac{(\arcsine \sqrt{P_1}) - (\arcsine \sqrt{P_2})}{\sqrt{(820.8(1/n_1) + (1/n_2))}}$$

where P_1 and P_2 are percent frequencies and n_1 and n_2 are respective sample sizes. 820.8 is a constant representing the parametric variance of a distribution of arcsine transformations of percentages. The t_s values were compared to critical values of Student's t distribution (two-tailed). In addition to performing 2x2 G tests, data were also analyzed using the software Epi Info™ 6.0 (Center for Disease Control and Prevention, Atlanta, GA, USA), which utilized chi-square analysis, to ensure accuracy of the statistical results obtained.

Results

A total of 291 oocytes were collected from 10 females that underwent Regimen A (full stimulation cycle of r-FSH and r-hCG), averaging 19 ± 10 oocytes per cycle. Of these, 166 were mature upon collection (maturation rate of 57 ± 12 sd% across collections). Immature oocytes obtained from Regimen A cycles matured *in vitro* (82/125) at a rate of 62 ± 26 % across collections. A total of 192 oocytes were collected from the same 10 females after Regimen B stimulation cycles (r-FSH-only), averaging 16 ± 8 oocytes per cycle. Of these, 156 metaphase II

oocytes were obtained after IVM overnight (maturation rate $80\pm 9\%$). There was no significant difference in terms of numbers of oocytes collected (mean \pm sd) from Regimen A and B nor was there a significant difference in IVM rate for Regimen A and B immature oocytes. With the exception of occasional loss during fixation and preparation of samples and early attempts to incorporate IVF into the experimental plan, all of the resulting mature oocytes were divided fairly evenly within a cohort between the two fixation protocols.

Investigation into rate of aneuploidy

Variables studied included IVO vs. IVM and young (5-15 yrs) vs. geriatric (>17 yrs) females. Chromosome spreads of oocytes within each category were examined (Figure 4.1). Because the fixation technique could result in artefactual loss of chromosomes through excess spreading (Kamiguchi *et al.*, 1993; Pellestor *et al.*, 2006), a conservative estimate of aneuploidy would only include incidences of hyperhaploidy and was reported as such here. However, although artificial loss of chromosomes could certainly be the cause of most observed hypohaploidies, it must be noted that various mechanisms such as anaphase lag (Martin, 1984; Coonen *et al.*, 2004) and displacement of chromosomes (Ford and Lester, 1982; Williams and Fisher, 2003) could also explain the loss of chromosomes in female meiosis and must be considered. Although it has not been definitively demonstrated, theoretically the process of non-disjunction should produce an equal number of hypo- and hyperhaploid oocytes. Therefore, another approach for estimating aneuploidy rate is to double the number of observed hyperhaploidies. This approach was also reported here as total aneuploidy rate. Both estimates reported, i.e., the strict hyperhaploidy rate and total aneuploidy rate (2 x hyperhaploidy) included frequencies of extra whole chromosomes and/or single chromatids.

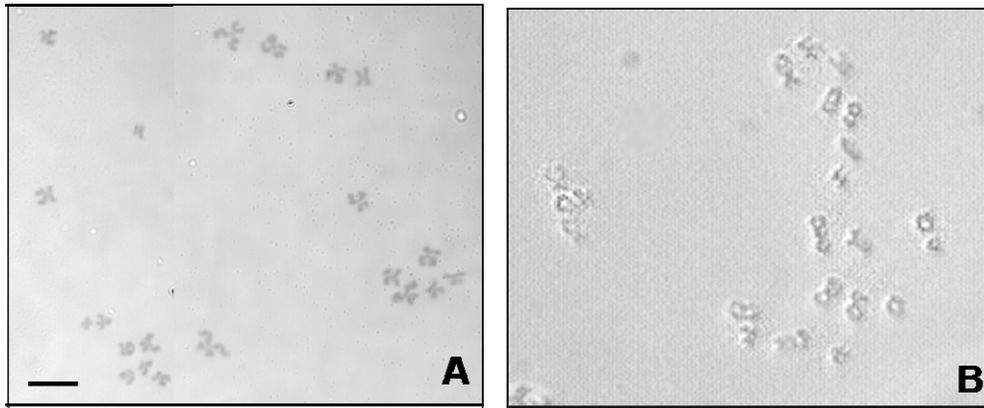


Figure 4.1. Chromosome spreads of metaphase II rhesus macaque oocytes representing (A) a normal karyotype (21 chromosomes) and (B) hyperhaploid karyotype (22 chromosomes). Bar represents 10 μ m.

Table 4.1. Chromosomal analysis of IVO and IVM oocytes .

Maturation Condition	A1	A2	B
Oocytes fixed	59	37	77
Oocytes nonanalyzable	8	4	9
Chromosomes clumped	5(62.5%)	2(50.0%)	5(55.6%)
Chromosomes lost	3(37.5%)	2(50.0%)	4(44.4%)
Oocytes analyzed	51	33	68
Haploids	42(82.4%)	26(78.8%)	47(69.1%)
Hypohaploids	6(11.8%)	3(9.1%)	9(13.2%)
Hyperhaploids	3(5.9%)^a	4(12.1%)^{ab}	12(17.6%)^b
2 x Hyperhaploids	6(11.8%)^a	8(24.2%)^{ab}	24(35.2%)^b

Condition A1 = IVO oocytes from Regimen A (full stimulation cycle, r-FSH and r-hCG). Condition A2 = subclass of IVM oocytes from Regimen A. Condition B = IVM oocytes from Regimen B (r-FSH-only). N = 10 females. For a given estimate (hyperhaploids; 2 x hyperhaploids), different letters indicate significant differences among maturation conditions ($p < 0.05$). Numbers reported include samples from both young and geriatric females.

A total of 51 IVO oocytes were analyzed from 10 females that underwent Regimen A stimulation (Table 4.1). The conservative estimate of hyperhaploidy was 5.9%, while the total aneuploidy rate was 11.8%. Of the 33 oocytes that were not mature upon collection after Regimen A and underwent IVM, hyperhaploidy was 12.1% (total aneuploidy rate 24.2%). A

total of 68 IVM oocytes from Regimen B cycles were analyzed. Hyperhaploidy was 17.6% (total aneuploidy rate 35.2%). There were no significant differences in hyperhaploidy or total aneuploidy rate between classes of oocytes collected from Regimen A (IVO and IVM oocytes) or between IVM oocytes from Regimen A and B. However, there was a significant difference in rate of hyperhaploidy ($p = 0.04$) and total aneuploidy ($p = 0.003$) between IVO oocytes collected from Regimen A cycles and IVM oocytes from Regimen B cycles. Sample oocytes from both young and old females were pooled in the above analyses.

Table 4.2. Chromosomal analysis of IVO and IVM oocytes from young and old females.

Maturation Condition	A1		A2		B	
	young	Old	Young	Old	Young	Old
Oocytes analyzed	32	19	21	12	49	19
Haploids	24(75.0%)	14(73.7%)	17(81%)	9(75.0%)	33(67.3%)	14(73.7%)
Hypohaploids	3(9.4%)	3(15.8%)	2(9.5%)	1(8.3%)	8(16.3%)	1(5.3%)
Hyperhaploids	1(3.1%)	2(10.5%)	2(9.5%)	2(16.7%)	8(16.3%)	4(21.1%)
2 x Hyperhaploids	2(6.3%)^a	4(21.1%)^{ab}	4(19.0%)^{ab}	4(33.3%)^b	16(32.7%)^b	8(42.1%)^b

Condition A1 = IVO oocytes from Regimen A (full stimulation cycle, r-FSH and r-hCG). Condition A2 = subclass of IVM oocytes from Regimen A. Condition B = IVM oocytes from Regimen B (r-FSH-only). N = 10 females, 7 young and 3 old females. For a given estimate (hyperhaploids; 2 x hyperhaploids), different letters indicate significant differences among maturation conditions ($p < 0.05$).

Since numbers of oocytes collected from geriatric females were markedly lower than from young females, the sample size for this subclass was low. Nevertheless, treatment groups were further divided by age (Table 4.2). Regimen A IVO oocytes ($n = 32$) from 7 young females exhibited a hyperhaploidy rate of 3.1% (total aneuploidy rate 6.3%), while oocytes from 3 geriatric females in that group ($n = 19$) exhibited rates of 10.5% hyperhaploidy (total aneuploidy rate 21.1%). Regimen A IVM oocytes from young females ($n = 21$) displayed a hyperhaploidy

rate of 9.5% (total aneuploidy rate 19%) and oocytes (n = 12) from older females were 16.7% hyperhaploid (total aneuploidy rate 33.3%). Regimen B IVM oocytes (n = 49) from young females were 16.3% hyperhaploid (total aneuploidy rate 32.7%) while oocytes (n = 19) from older females in this group were 21.1% hyperhaploid (total aneuploidy rate 42.1%). There was no significant difference between age groups within any of the maturation conditions examined. However, there were significant differences in total aneuploidy rates (but not hyperhaploidy rates) between Regimen A IVO oocytes from young females and Regimen B IVM oocytes from both young ($p = 0.005$) and old females ($p = 0.005$)(6.3% vs. 32.7% and 42.1%, respectively). Additional samples might provide more conclusive results.

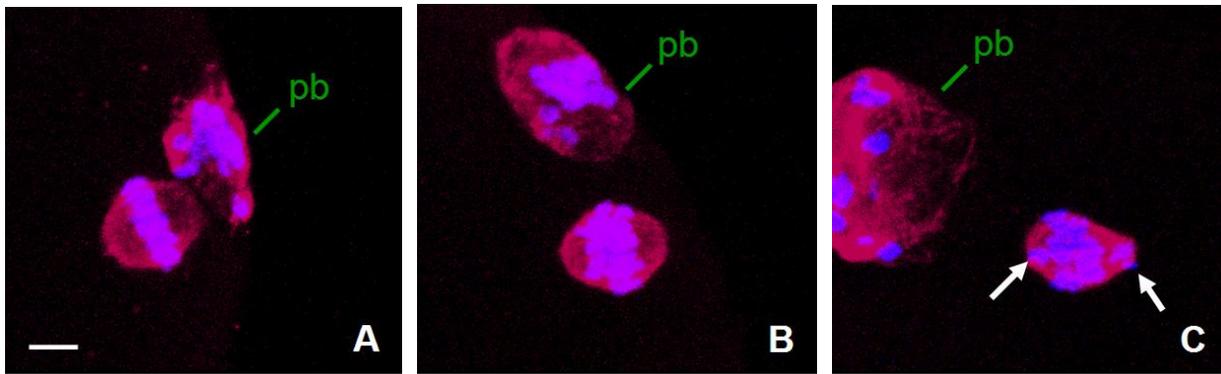


Figure 4.2. Merged immunofluorescent images of oocyte spindles and chromosomes. **(A, B)** Samples obtained from Regimen A cycles (full stimulation, r-FSH and r-hCG) in young females, i.e., IVO oocytes. **(A)** Normal metaphase II oocyte displaying barrel-shaped spindle and chromosomes aligned on the spindle equator. In contrast, spindle and chromosomes in the polar body are disorganized. **(B)** Metaphase II oocyte that appears to display unorganized chromosome alignment around the spindle equator in a 2-dimensional image. Use of confocal microscopy provided a 3-dimensional image demonstrating that this oocyte was actually normal in both spindle morphology and chromosome alignment. **(C)** Sample obtained from a Regimen B cycle (FSH-only) from a geriatric female, i.e., IVM oocyte. Spindle was malformed with a tear-drop shape (seen in confocal 3-D image) with evidence of chromosome lag (arrows). “pb” indicates polar body. Bar represents 10 μ m.

Table 4.3. Meiotic spindle analysis of IVO and IVM oocytes.

Maturation Condition	A1	A2	B
Oocytes analyzed	53	34	64
Normal metaphase II oocytes	40(75.7%)	18(52.9%)	32(50.0%)
Abnormal metaphase II oocytes	13(24.5%) ^a	16(47.1%) ^b	32(50.0%) ^b
Abnormal spindle*	1(1.9%)	0	2(3.1%)
Abnormal chromosome alignment*	4(7.5%) ^a	9(26.5%) ^b	21(32.8%) ^b
Abnormal spindle and chromosome alignment*	4(7.5%)	6(17.6%)	8(12.5%)
Other anomalies*	4(7.5%)	1(2.9%)	1(1.6%)

Condition A1 = IVO oocytes from Regimen A (full stimulation cycle, r-FSH and r-hCG). Condition A2 = subclass of IVM oocytes from Regimen A. Condition B = IVM oocytes from Regimen B (r-FSH-only). N = 10 females. (*) Percentages in these categories represent the rate at which these abnormalities occurred within the total population analyzed. For a given abnormality, different letters indicate significant differences among maturation conditions ($p < 0.05$). ‘Other anomalies’ refers to architecture resembling telophase rather than metaphase. Numbers reported include samples from both young and geriatric females.

Of the 53 IVO oocytes from Regimen A, 24.5% exhibited abnormal metaphase II characteristics. This was significantly lower than the IVM oocytes collected from the same full stimulation cycles (47.1% abnormal) ($p = 0.03$) and IVM oocytes from Regimen B (50% abnormal) ($p = 0.005$). Further analysis demonstrated that the difference could be attributed to the significant increase in rate of abnormal chromosome alignment on the metaphase spindle in IVM oocytes originating from either stimulation regimen (7.5% vs. 26.5% and 32.8%) ($p = 0.02$ and $p = 0.001$, respectively). Samples from young and old females were pooled in these calculations.

Table 4.4. Meiotic spindle analysis of IVO and IVM oocytes from young and old females.

Maturation Condition	A1		A2		B	
	young	Old	young	old	young	Old
Oocytes analyzed	32	21	26	8	45	19
Normal metaphase II oocytes	25(78.3%)	15(71.4%)	14(53.8%)	4(50.0%)	26(57.8%)	6(31.6%)
Abnormal metaphase II oocytes	7(21.9%) ^a	6(28.6%) ^a	12(46.2%) ^{ab}	4(50.0%) ^{ab}	19(42.2%) ^{ab}	13(68.4%) ^b
Abnormal spindle*	0	1(4.8%)	0	0	2(4.4%)	0
Abnormal chromosome alignment*	2(6.3%) ^a	2(9.6%) ^{ab}	6(23.1%) ^{ab}	3(37.5%) ^b	11(24.4%) ^b	10(52.6%) ^c
Abnormal spindle and chromosome alignment*	2(6.3%)	2(9.6%)	5(19.2%)	1(12.5%)	6(13.3%)	2(10.5%)
Other anomalies*	3(9.4%)	1(4.8%)	1(3.8%)	0	0	1(5.3%)

Condition A1 = IVO oocytes from Regimen A (full stimulation cycle, r-FSH and r-hCG). Condition A2 = subclass of IVM oocytes from Regimen A. Condition B = IVM oocytes from Regimen B (r-FSH-only). N = 10 females, 7 young and 3 old females. (*) Percentages in these categories represent the rate at which these abnormalities occurred within the total population analyzed. For a given abnormality, different letters indicate significant differences among maturation conditions ($p < 0.05$). 'Other anomalies' refers to architecture resembling telophase rather than metaphase.

No differences were evident among oocytes from young females in any of the maturation conditions when total occurrence of abnormal metaphase II oocytes was considered. There was a significant difference in total occurrence of abnormal metaphase II oocytes between IVO oocytes from old females in Regimen A and IVM oocytes from old females in Regimen B ($p = 0.02$). When specific abnormalities were investigated, significant differences were demonstrated in abnormal chromosome alignment. Specifically, Regimen B IVM oocytes from old females displayed abnormal chromosome alignment on the spindle equator at a significantly higher rate than oocytes of any other condition or age group ($p < 0.05$). Also, IVO oocytes from young females showed this anomaly at a significantly lower rate ($p = 0.04$) than Regimen B IVM oocytes from young females. It should be noted that several of the statistical comparisons approached significant difference (e.g. young IVO oocytes vs. young IVM oocytes from Regimen A), i.e., p value of 0.06. An increase in sample size would be necessary to clearly distinguish if there is a significant difference.

Discussion

Results from this study support the observation that there are differences between IVO and IVM primate oocytes in the frequencies of aneuploidy and abnormal meiotic spindle arrangement. An *in vitro* culture environment, while producing what may appear to be morphologically normal mature oocytes (i.e., extrusion of the first polar body along with uniform cytoplasm), does not necessarily generate a “normal” oocyte in primates. Previous studies in primates have demonstrated a correlation between site of oocyte maturation and developmental capacity (Lanzendorf *et al.*, 1990; Schramm and Bavister, 1999). *In vivo* matured oocytes fertilize and develop *in vitro* at a relatively high rate, with 37% reaching the blastocyst stage

versus only 7% of IVM oocytes from r-FSH-only cycles (Schramm and Bavister, 1999). Slower-maturing oocytes from full stimulation cycles, i.e., those that were immature upon aspiration, also display a decreased capacity for successful development after fertilization (Lanzendorf *et al.*, 1990). It may be that slower maturing oocytes are fundamentally compromised or that the final stages of maturation *in vitro* affect their developmental potential.

From a practical viewpoint, the likely sources of IVO and IVM oocytes are those retrieved from natural cycles and ovariectomies or necropsies, respectively. However, these samples are difficult to obtain, require a considerable amount of time to accumulate a sufficient amount of data, and, in the case of necropsies, are sporadic in nature. Therefore, in this study, IVO oocytes were obtained from females stimulated with r-FSH to recruit multiple ovarian follicles together with r-hCG to induce IVO. Oocytes that were immature upon collection after full stimulation underwent IVM and represented a subclass of Regimen A oocytes. Since this subclass of oocytes had failed to undergo IVO, they were presumably defective. *In vitro* matured oocytes were also obtained from females stimulated with r-FSH only.

Unlike inbred mice, monkeys are a genetically heterogeneous population although the CPRC colony may be more homogeneous than populations at other primate centers, but nevertheless genetically different from those populations (see Chapter 3). The genetic variability of the monkeys was compensated by experimental design because the same 10 females were used to provide oocytes for both treatments.

This study would have ideally included investigation of developmental competence of cohort oocytes using IVF and culture of embryos to the blastocyst stage. Although birth of live offspring after embryo transfer is the ultimate demonstration of oocyte competence, this technique is too inefficient in monkeys to be used as a quantitative experimental endpoint. Even

restricting studies to *in vitro* development, each cohort of oocytes would need to be split evenly into three groups for analysis of karyotype, spindle morphology and developmental potential. However, given the low numbers of oocytes collected per aspiration for the females allocated to this study (averaging 18 ± 9 /collection with IVO and IVM rates of $57 \pm 8\%$, $66 \pm 16\%$, and $81 \pm 9\%$) and the destructive nature of the karyotype and spindle analyses, it was not possible to split the cohorts into three groups. Furthermore, it would have been impossible to include the developmental analysis because of time and manpower constraints.

Recently, a non-invasive, non-destructive method of visualizing oocyte spindles has been developed using a polarized light microscope, the “PolScope”, which relies on the birefringent optical properties of the spindle. The PolScope is not as sensitive as confocal immunostaining in detecting chromosome misalignment, but can provide comparable information regarding spindle structure (Keefe *et al.*, 2003). While the ability to non-destructively image oocyte spindles would be ideal, especially when coupled with IVF and embryo culture to investigate developmental competence, this resource was not available for this work. Instead, oocytes were fixed and stained for specific spindle structures (microtubules, microfilaments and chromatin) and observed using confocal microscopy. This technology, while destructive, enables the visualization of structures in 3-dimensions, providing a more complete image of a sample and allowing the investigator to detect errors that are inherent in 2-dimensional epifluorescent imaging. Figure 2B is a good example of an image that could have easily been misinterpreted as having scattered chromosomes if only viewed in 2 dimensions.

Yin *et al.* (2006) also investigated spindle morphology in a macaque species as part of their efforts to study the effect of priming with r-hCG on developmental competence. They found a very high incidence of anomalies: less than half of the samples had a normal spindle and

only a third had correctly aligned chromosomes. They state that images were examined with a Nikon fluorescence microscope, which, as stated earlier, only allows visualization in 2 dimensions. It is difficult to make a definitive judgment due to low quality of images provided in their manuscript, but without being able to see a spindle in 3 dimensions, it is quite possible to mistakenly interpret flat spindle images from various angles as being anomalous and so their results must be interpreted with caution. In another study, Li *et al.* (2006b) investigated incidences of spindle aberrations in oocytes from polycystic ovary syndrome (PCOS) patients that underwent IVO or IVM. Their results demonstrated a significant difference in both spindle configuration and chromosome configuration in IVO and IVM oocytes (13.6% vs. 43.7% and 9.1% vs. 33.3%, respectively). This report was based on a small sample size (IVO, n = 22 and IVM, n = 48) and, more importantly, because these oocytes originated from females with PCOS, it is likely these oocytes were already inherently compromised.

In the present study, immunostaining and confocal technology demonstrated that chromosome congression on the metaphase II spindle was significantly affected by maturation conditions, an anomaly that was even more pronounced in oocytes obtained from older females (Table 4.4). Among Regimen B oocytes, 52.6% (n = 19) from old females exhibited spindles with chromosomes scattered from the spindle equator. *In vitro* matured Regimen A oocytes from old females also displayed a high rate of aberrant chromosome alignment (37.5%, n = 8). The limited sample size should be noted for both of these study groups. In comparison to faulty chromosome congression, incidences of abnormal spindle structure were less common in my study samples, perhaps indicating that while spindle microtubules were competent to form normal barrel-shaped spindles, mechanisms involved in attaching and arranging chromosomes on the spindle equator were affected.

Incidences of aberrant chromosome alignment on the spindle equator may be linked to disturbances of cytoplasmic components, such as expression of the protein XKLP1, which is involved in chromosome congression (Brunet and Maro, 2005), and MTOC activity during spindle assembly (Sanfins *et al.*, 2003). On the other hand, oocytes do possess a checkpoint regulatory pathway. Regulatory components of this checkpoint system include the genes Bub 1 (Budding uninhibited by benzimidazole 1) (Tsurumi *et al.*, 2004) and Mad2 (Mitotic arrest-deficient 2) (Wassman *et al.*, 2003; Tsurumi *et al.*, 2004; Homer *et al.*, 2005) which encode kinetochore-associated proteins believed to ensure proper chromosome segregation by delaying anaphase onset until all chromosomes are correctly attached to the spindle through their kinetochores (chromosomal attachment points for spindle fibers located within the chromosome centromeres) (Steuerwald *et al.*, 2001). With this checkpoint system in place, it is possible that the incidences of chromosome congressional failure seen in fixed samples in this study and in others may be corrected over time. However, expression of spindle assembly checkpoint components are found to degrade with maternal age and may be a factor in reported age-related aneuploidy.

Aneuploidy can result from premature disjunction or non-disjunction of homologous chromosomes during meiosis I, resulting in extra or missing chromosomes in metaphase II oocytes. It can also arise from the premature separation of chromatids during meiosis I, resulting in extra or missing single chromatids in metaphase II oocytes. In this study, whole chromosome hyperhaploidy was observed at a higher frequency (84% of hyperhaploid oocytes) than single chromatid hyperhaploidy (16%), which indicates premature disjunction or non-disjunction of homologous chromosomes as the most common cause of aneuploidy. The rate of total aneuploidy (2 x hyperhaploidy) in the current study was significantly increased in Regimen A

IVM oocytes from old females and Regimen B IVM oocytes from both young and old females, which may possibly have resulted from degradation of the spindle assembly checkpoint system during meiosis I. Schramm *et al.* (2002) also investigated aneuploidy in rhesus monkey oocytes in relation to female age, but were not able to make a definitive statement due to a limited dataset, i.e., 14 analyzable oocytes from 5 young females (2 x hyperhaploidy rate of 0%) and 16 analyzable oocytes from 6 older females (2 x hyperhaploidy rate of 14.3%). The conservative hyperhaploidy rate in IVM oocytes regardless of female age in my study (12.1% of Regimen A oocytes, 17.6% of Regimen B oocytes) was comparable to reported hyperhaploidy rates from other mammalian species: bovine, 7.1% (Lechniak and Switonski, 1998); pig, 11.9-21.6% (Koenig and Stormashak, 1993); and horse, 5.5% (King *et al.*, 1990). As is often the case in aneuploidy investigations, these rates were based upon small sample sizes and an increase in sample numbers may reveal quite different results.

In summary, this study demonstrated IVO oocytes to be the most meiotically competent, regardless of female age. I had suspected that Regimen A IVM oocytes were inherently flawed because of their immature state upon collection after full stimulation cycles. Interestingly, only Regimen A IVM oocytes from geriatric females significantly differed in total aneuploidy rate and metaphase II architecture from young female IVO oocytes. Immature oocytes from young females exposed to a full stimulation regimen may possess some intrinsic quality which allows them to recover or tolerate disturbances that may be introduced through IVM. This “quality” may be absent in older female oocytes due to degradation of cytoplasmic components over time, e.g., spindle assembly checkpoints. Not surprisingly in view of their lower rates of developmental competence in previous studies (Schramm and Baviser, 1996b; Zheng *et al.*, 2001b), oocytes that were not exposed to r-hCG during ovarian stimulation and were matured *in*

vitro displayed the highest rates of hyperhaploidy and abnormal metaphase spindle and chromosome alignment.

In vitro maturation presents a challenge for assisted reproductive technologies. *In vitro* maturation can induce meiotic anomalies in the macaque oocyte, especially those obtained from older females. The results from this study provide possible explanations to account for the reported reduction in developmental competence of IVM versus IVO primate oocytes. Investigations into the relationship between nuclear maturation (spindle organization, chromosome segregation) and cytoplasmic maturation are key to designing successful IVM protocols. This study provided information on the nuclear aspects of IVM, namely the effect of maturation conditions and age on incidences of abnormal chromosome segregation during meiosis I and abnormal chromosome congression during meiosis II, which can be utilized to determine optimal IVM conditions and possibly improve cytoplasmic maturation as well. Future research should include investigation into the developmental capacity of cohort oocytes as well as meiotic anomalies. These efforts could be greatly aided by use of the PolScope mentioned earlier because it is a non-destructive tool for providing information on spindle architecture within an oocyte that can subsequently be fertilized *in vitro*.

At present, application of IVM technology to conservation of endangered species is not considered a viable option because of its challenging nature. As this technique improves through investigations into basic characteristics of oocyte maturation, such as in the current study, captive breeding programs may allow reproductive biologists to access their animals to recover oocytes from individuals that are of genetic value and/or may not be well-represented in the captive population gene pool. This may be possible through natural cycle monitoring and retrieval or possibly gonadotropin stimulation. Either of these approaches will surely be difficult

to apply to captive populations of animals, but not so difficult that they should not be considered, as studies with captive Felidae have shown (Pope 2000; Pope *et al.*, 2006).

Chapter 5

Comparison of Protocols for Cryopreservation of Rhesus Monkey Spermatozoa by Post-Thaw Motility Recovery and Hyperactivation

Introduction

Original experimental plans for the analysis of oocyte quality included investigating developmental competence. To accomplish this goal, it would be beneficial to have a readily available supply of viable spermatozoa for use in *in vitro* fertilization (IVF) experiments. Collecting semen as needed imposes time constraints on the investigators because concomitant oocyte collection and processing is very laborious; in addition, male donors may be unnecessarily subjected to frequent collection protocols. In contrast, cryopreservation of semen offers greater convenience for IVF experiments and is crucial for gene banking of genetically valuable animals. Cryobanking of spermatozoa can also reduce the frequency of ejaculation collections, reducing stress on the animals and making more efficient use of each semen sample collected. When the same semen sample can be used for several experiments, the power of experimental design is increased by reducing between-sample variability. A number of techniques have been described for cryopreserving spermatozoa of various non-human primate species (baboon: Kraemer & Vera Cruz, 1969; squirrel monkey: Lawrence *et al.*, 1976; and cynomolgous monkey: Mahone & Dukelow, 1978; Tollner *et al.*, 1990; Sankai *et al.*, 1994). The purpose of the present study was to evaluate the effectiveness with the rhesus macaque of several published primate sperm cryopreservation protocols, including two techniques that were not developed with macaques, to determine the most efficient one. The rhesus macaque is the most widely studied non-human primate species for biomedical research. A reliable method for cryopreservation of spermatozoa from this species would not only contribute to basic

embryological research, but could also be extrapolated to other non-human primate species that are considered threatened or endangered. The effectiveness of each method was compared by recording post-thaw sperm longevity (motility % and duration) and ability of spermatozoa to exhibit hyperactivated motility in response to chemical activators (caffeine, dbcAMP) needed for rhesus sperm *in vitro* to attain the ability to fertilize eggs, a process termed “capacitation” (Boatman & Bavister, 1984). Hyperactivated motility is a definitive endpoint for capacitation (Katz *et al.*, 1989; Yanagimachi, 1994). Thus, the ability to hyperactivate provides a reasonable assessment of the fertilizing capability of spermatozoa prior to performing IVF. Because of the number of treatments used in this study, and the limited numbers of eggs and operator time, it was impractical to use IVF as an endpoint for all cryopreservation methods.

Materials and Methods

All animals were used with approval of the Tulane National Primate Research Center and the University of Puerto Rico’s Caribbean Primate Research Center (CPRC) Institutional Animal Care and Use Committees. Males used in the study had been previously trained for pole-and-collar-chair restraint. Ejaculates were obtained via penile electroejaculation (details are as described in Gould & Mann, 1988) on three occasions each from three different males for a total of 9 collections. Samples from each male were collected on different days. Each ejaculate was divided into four treatment groups. Progressive motility of spermatozoa before and after addition of the semen extender for each treatment was evaluated subjectively by light microscopy and expressed as % progressively motile. Each treatment was executed according to its published protocol as summarized below. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Method A

Method A (Si *et al.*, 2000) was developed for rhesus monkey spermatozoa. Freezing medium was prepared according to published protocol for “TTE” solution (Sankai *et al.*, 1994), including use of fresh egg yolk and the sugars glucose and lactose. Egg yolk was centrifuged for 1 hr at 4°C to separate yolk droplets and the supernatant was added to the freezing medium. Following 2 washes of a semen sample with TALP-Hepes medium (Bavister *et al.*, 1983), the sperm cell suspension was diluted with 9 volumes of TTE without glycerol at approx. 22°C. The diluted sample was cooled to 4°C over 2 hr by submerging the sample tube in a pre-cooled glass beaker full of water. Sperm samples were further diluted at this low temperature by stepwise addition with an equal volume of pre-cooled TTE containing 10% glycerol for a final glycerol concentration of 5% (v/v). The extended glycerolated sperm suspension was drawn into pre-cooled plastic cryostraws (1/4 cc size; Agtech, Inc., Manhattan, KS, USA), which were then sealed with Critoseal capillary tube sealant (Fisher Scientific, Hampton, NH, USA). The straws were suspended 10 cm above the surface of liquid nitrogen for 10 min then directly plunged into the liquid nitrogen for storage. Straws were kept in liquid nitrogen for at least 7 days before being thawed. Sperm samples were rapidly thawed by submersion in a 37°C water bath for 2 min and subsequently washed with warmed TALP-Hepes in accordance with the originally published protocol.

Method B

Method B (Sanchez-Partida *et al.*, 2000) was developed for the rhesus monkey. Semen samples were diluted five-fold at 30°C with a TRIS-based medium containing glycerol (final concentration of 6%), egg yolk and glucose. The diluted samples were then slowly cooled to

5°C in the refrigerator (~2 hr). Aliquots of the cooled sample were frozen in pellet form on dry ice (~1 min), then stored in cryovials that were placed directly into liquid nitrogen for storage. To thaw samples, individual pellets were placed in dry test tubes that were shaken while submerged in a 37°C water bath until completely thawed (about 1-2 minutes) in accordance with the original protocol.

Method C

Method C (Seier *et al.*, 1993) was developed for vervet monkey spermatozoa. The semen samples were diluted 1:1 with unglycerolated extender, which included egg yolk, glucose, and TES and TRIS buffers, at 32°C. The sample was then diluted at 32°C with an equal volume of extender containing 10% glycerol for a final glycerol concentration of 5%. The glycerolated sample was cooled to 5°C over 30 min by submerging the sample tube in a pre-cooled glass beaker full of water. The extended glycerolated semen was drawn into pre-cooled plastic cryostraws (1/4 cc size; Agtech, Inc.) and sealed with Critoseal (Fisher Scientific). The straws were suspended 2 cm above liquid nitrogen for 20 min prior to direct plunging into liquid nitrogen for storage. Thawing of individual straws was performed by placing them in a beaker full of water at 32°C for one min. according to the original published protocol.

Method D

Method D (Isachenko *et al.*, 2005) was developed for humans. This method does not employ a cryoprotectant, relying on very rapid freezing and warming for sperm survival. The original work compared results of the non-cryoprotectant technique using various storage forms/apparati. The current study utilized the droplet method because this was convenient and

was not found to significantly differ in motility recovery rate from the other forms (cryoloops, open-pulled and open-standard straws) reported. Semen samples were washed 2 times using warmed (37°C) TALP-Hepes medium and then suspended in ~300 µl of the same medium. Aliquots of the sperm sample were frozen by dropping them onto aluminum foil that was pre-cooled in liquid nitrogen vapors to -160°C (~5 min). The frozen droplets were placed in cryovials then plunged into liquid nitrogen for storage. For thawing, individual droplets were quickly removed and placed into pre-warmed (37°C) TALP-Hepes medium and agitated until thawed (~1 min) in accordance with the originally published protocol.

For each cryopreservation method, thawed semen aliquots were divided into one of three incubation conditions: (1) incubation in pre-equilibrated TALP medium (37°C, 5% CO₂ in air) with no chemical activators; (2) incubation in pre-equilibrated TALP medium at (37°C, 5% CO₂ in air) with chemical activators; and (3) incubation on the bench-top in TALP-Hepes medium at approximately 22°C. Small samples were taken from each treatment and observed for progressive motility at 0, 1, 2, 4 and 24 h post-thaw. These times were chosen because sperm survival for at least 4 h is necessary for sperm capacitation to take place prior to egg penetration during IVF. The duration of post thaw motility in the original reports was not reported, so we did not know how long to expect sperm survival to last using the four methods. We chose 24 h as an arbitrary endpoint. Sperm motility recovery rate was assessed using the formula (post-thaw motility % X 100)/prefreeze motility % (Younis *et al.*, 1998). A portion of each frozen-thawed sample was exposed to chemical activators (caffeine and dbcAMP, each at 1mM final concentration) ~30 min post-thaw in order to determine the ability of the spermatozoa to hyperactivate. Hyperactivated motility is characterized by a “whiplash” motion, or high

amplitude flagellar bending in a figure 8 pattern, and is usually qualitatively assessed via visualization by a trained observer, as in the present study. Although this assay is subjective, with practice this analysis yields reproducible results (Bavister & Andrews, 1988). Statistical analysis of sperm motility recovery rates per method and time point was performed using 2x2 G tests as previously described (Chapter 4).

Results

No samples frozen using the method developed for human spermatozoa (Method D) survived the freeze-thaw process (motility = 0 at time point 0 post-thaw) so they are not included in Figures 1 and 2. Post-thaw motility recovery rates for Methods A-C in the present study were reasonable (68%, 73%, and 62%, respectively) and similar to the results reported in the original studies. The majority (75% or higher) of motile spermatozoa from each of these methods underwent hyperactivation within 30-60 min upon exposure to chemical activators. Motility decreased over time in culture within each treatment, regardless of incubation conditions. When incubated at 37°C under 5% CO₂ in air, spermatozoa that had been frozen using Method A were significantly less motile at 4 and 24 h than with Methods B and C, and Method B spermatozoa were significantly less motile at 24 h than those treated with Method C (Figure 5.1). Method A spermatozoa incubated at 22°C were also significantly less motile at 24 h than Methods B and C spermatozoa held at this temperature (Figure 5.2). Overall, Method C best preserved sperm motility over time within each incubation condition. Incubation at 22°C preserved motility better than incubation at 37°C.

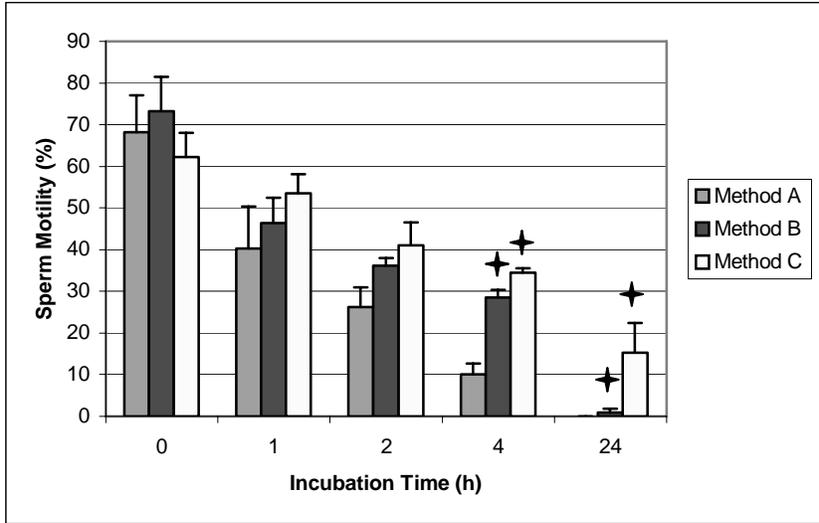


Figure 5.1. Comparison of rhesus macaque sperm motility recovery following cryopreservation of spermatozoa using various methods. Thawed samples were incubated in pre-equilibrated (37°C, 5% CO₂ in air) TALP medium. Data are means ±SD for 9 ejaculates (3 ejaculates per male), each frozen using 3 methods (spermatozoa treated with Method D did not survive). (✦) indicates significant difference in sperm motility recovery rate among the samples at specific incubation time points.

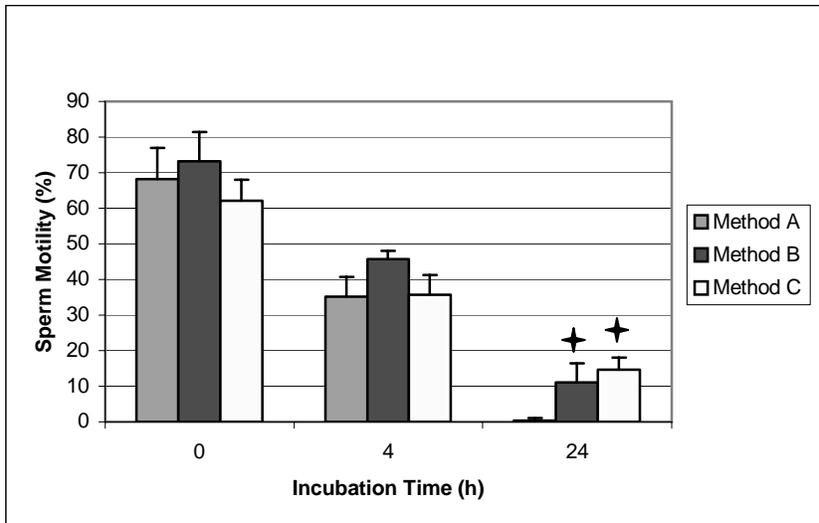


Figure 5.2. Comparison of rhesus macaque sperm motility recovery following cryopreservation of spermatozoa using various methods. Thawed samples were incubated in TALP-Hepes medium at 22°C. Data are means ±SD for 9 ejaculates (3 ejaculates per male), each frozen using three methods. (✦) indicates significant difference in sperm motility recovery rate among the samples at specific incubation time points.

Discussion

Cryopreservation methods developed for non-human primates (rhesus and vervet monkeys, Methods A-C) all yielded good immediate post-thaw recovery of progressively motile spermatozoa, and acceptable motility at 4h post-thaw, despite the differences among their protocols. These results agree with those of Li *et al.* (2006a) who reported no significant differences in initial motility recovery rate, or in plasma membrane and acrosome integrity, after use of various extenders in macaque species. With the exception of Method D, all of the methods investigated in the current study would be suitable for short-term use such as for IVF, and perhaps also for artificial insemination (AI) procedures, if used within a few hours post-thaw. If used for these purposes, these methods would also be suitable for storing or gene banking of rhesus spermatozoa. Since the majority of spermatozoa cryopreserved with these three methods underwent hyperactivation following exposure to chemical activators, it can be assumed that each method would provide spermatozoa capable of oocyte penetration and fertilization (Boatman & Bavister, 1984; Katz *et al.*, 1989; Yanagimachi, 1994). In contrast, Method D is completely inappropriate for rhesus macaque sperm cryopreservation because it consistently killed all spermatozoa subjected to this treatment. It is important to note that this method was developed for human sperm cryopreservation and the results reported here may be due to fundamental differences in human and macaque spermatozoa. Method D does not employ a cryoprotectant during the freezing process and rhesus macaque spermatozoa may require one to be present during freezing because they are more susceptible to cryo-damage than human spermatozoa.

In situations where multiple IVF or AI cycles are scheduled during a limited time span (i.e., 4h), Methods B and C appear optimal if spermatozoa are incubated at room temperature post-thaw. Maintaining frozen-thawed spermatozoa at 22°C instead of 37°C may preserve their integrity/life span by lowering metabolic activity and preventing rapid accumulation of

metabolic waste products such as lactic acid (Jones & Bavister, 2000). Although Method A utilizes two sugars - glucose (monosaccharide) and lactose (disaccharide) - and Methods B and C utilize only glucose in their cryoprotectant media, the addition of disaccharide may not be a factor influencing motility rate differences among these methods. A recent study by Si *et al.* (2006) indicated that glucose alone versus both glucose and lactose in extender did not result in significant differences in initial motility recovery rate, or plasma membrane and acrosome integrity. One difference among Methods A-C that may be important is that Method A utilizes egg yolk centrifuged for 1 h at 4°C to separate yolk droplets, while in the other methods egg yolk is not centrifuged. Perhaps separation of the yolk droplets removes intrinsic components of yolk that would otherwise aid in long-term preservation of sperm motility.

The purpose of this study was to compare published methods for cryopreservation of primate spermatozoa. While three of the four methods examined in the current study performed well enough to support post-thaw sperm survival up to 4 hr, sufficient for IVF, survival at 24hr was low. Clearly, much more work needs to be done to improve cryopreservation protocols so that sperm survival is prolonged sufficiently that sequential IVF procedures can be performed. More efficient sperm preservation protocols would also contribute to the well-being of semen donors because the number of collections required for ART procedures could be reduced. Diluted ejaculates could be used for several experiments, eliminating possible male effects on the outcome of IVF procedures. The results presented indicate that to increase the usefulness of each sample, it would be beneficial to incubate thawed spermatozoa at 22°C after freezing with Method B or C. These techniques appear to be most appropriate for gene banking of rhesus semen, although their efficiency in preserving sperm function needs to be increased. Cryopreservation methods for rhesus monkey spermatozoa could also be applicable to other non-

human primate species, including endangered primates, in which semen collection may be more difficult, precluding optimization of semen cryopreservation protocols using these animals, and in which gene banking is of high importance.

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Chapter 6

Conclusions

Assisted reproductive technologies (ARTs), such as *in vitro* oocyte maturation (IVM) and *in vitro* fertilization (IVF), offer alternative approaches to species conservation by providing potential opportunities for reproduction in situations that may otherwise be fruitless. Although many of these ‘high-tech’ solutions have not yet been proven viable for pragmatic wildlife conservation, basic research and development of these emerging tools can provide necessary information needed to optimize these techniques and institute ART as a routine practice in both *ex situ* and *in situ* conservation efforts. A severely limiting factor in the successful application of ARTs is the availability of developmentally competent oocytes. Protocols have been developed in domestic and laboratory species to obtain mature, developmentally competent oocytes through exogenous hormone stimulation regimens, retrievals from natural estrous or menstrual cycles, or IVM techniques. The successful collection of *in vivo* matured (IVO) oocytes relies upon the ability to induce folliculogenesis with exogenous hormones and the ability to predict the optimum time for oocyte retrieval from preovulatory follicles. In contrast, retrieval of immature oocytes from antral follicles avoids these constraints and enables oocyte collection from prepubertal, pregnant or even dead animals.

To date, IVM does not have the same success rate across species studied. In primates in particular, IVM oocytes from unstimulated or from FSH-stimulated monkeys display reduced developmental competence compared with *in vivo* matured (IVO) primate oocytes (Schramm and Bavister, 1996a, 1996b; Gilchrist *et al.*, 1997; Zheng *et al.*, 2001a, 2001b, 2003) or IVM oocytes of other mammalian species (mice, Schroeder and Eppig, 1984; Eppig and Schroeder,

1989; sheep, Morton *et al.*, 2005; cattle, Coleman *et al.*, 2007). In this study I set out to investigate possible causes of reduced developmental capacity of primate oocytes using the rhesus macaque (*Macaca mulatta*) as my model.

This work began with an in-depth look at the quality of the monkey ovary over time in respect to morphology, building on sparse data available in previously published reports. The goal was to establish whether and when these long-lived animals experienced ovarian senescence. Sampling of females across an age range encompassing the entire reproductive spectrum (pre-pubescence to menopause) demonstrated an age-related pattern of follicle depletion similar to that described in the human ovary (Richardson *et al.*, 1987; Faddy *et al.*, 1992; Faddy, 2000). Changes in ovarian morphology included loss of stromal tissue and extremely low populations of primordial, primary and antral follicles, many of which had become atretic. These results indicated that attempts at oocyte recovery from older females (age 17+ years) would likely result in low numbers of oocytes, which might be developmentally compromised due to deterioration of companion somatic cells.

Following this study, I took part in collaborative efforts to establish an embryology laboratory at the Caribbean Primate Research Center (CPRC) in Puerto Rico and continue with my dissertation research into the reduced developmental capacity of primate oocytes. Many time-consuming difficulties were encountered along the way, including the establishment of a regular stimulation protocol and poor IVO rates. Retrieval results were improved by the end of the first reproductive season (May 2006) and I continued to collect data towards my dissertation aims. The main goal of the CPRC collaboration was to determine the effect of IVM and age on meiotic spindle integrity and chromosome segregation. Nuclear maturation anomalies are likely to occur as a result of maturation of oocytes *in vitro* and increasing age of females is known to be

associated with decreasing oocyte competence in women undergoing ART in infertility clinics. Although IVO and IVM oocytes would have been preferably recovered from natural cycles and ovariectomies or necropsies, respectively, these kinds of samples were not easily available. Therefore, IVO and IVM oocytes were obtained from stimulation cycles using r-FSH to recruit multiple follicles with or without the addition of r-hCG to induce final maturation. Each of the 10 females selected for this study underwent both of the treatments (r-FSH + r-hCG or r-FSH only) with at least one month's rest in between, in order to compensate for inherent heterogeneity of the population.

Immunostaining and confocal microscopy were utilized to visualize oocyte spindles as 3-dimensional images, reducing the possibility of misdiagnosis that may result from conventional 2-dimensional epifluorescent microscopy. Results demonstrated a significant increase in the incidence of faulty chromosome congression in relation to IVM and age of donor female. On the other hand, there were no significant differences in spindle morphology among the groups studied. Increased chromosome congressional anomalies may instead be attributed to disturbances of cytoplasmic components responsible for chromosome congression and segregation, such as the XKLP1 protein (Brunet and Maro, 2005) and checkpoint regulatory genes Bub 1 (Tsurumi *et al.*, 2004) and Mad2 (Wassman *et al.*, 2003; Tsurumi *et al.*, 2004; Homer *et al.*, 2005).

Results from chromosome spreads indicate a significant effect of IVM on the frequency of hyperhaploidy in metaphase II oocytes. The majority of hyperhaploidies were present as whole chromosomes (84% of hyperhaploidies), indicating that the most common cause of aneuploidy induced by IVM conditions in rhesus monkey oocytes was premature disjunction or non-disjunction of homologous chromosomes during meiosis I. Age of donor female also had an

effect of incidences of aneuploidy, but only if total aneuploidy rate (2 x hyperhaploidy rate) was considered. It should be noted that all of the above analyses relied upon relatively small sample sizes and an increase in N may provide alternative results.

Future studies would endeavor to combine meiotic spindle and chromosome analyses with developmental competence of embryos created from cohort oocytes and analyses of other components, such as mitochondrial DNA (mtDNA). Mitochondrial DNA is responsible for supplying sufficient ATP for transcription and translation during oocyte maturation. Spindle formation, checkpoint control and chromosome cohesion could be compromised by any aberration in mtDNA function. Mutations and deletions in mitochondria have been associated with age-related non-disjunction in human oocytes (Eichenlaub-Ritter *et al.*, 2003). Gibson *et al.* (2005) reported a three-fold increase in the frequency of mtDNA defects (i.e., common deletions) that appeared to be associated with the administration of exogenous r-FSH. High levels of exogenous FSH cause an increase in estrogen levels which may damage mitochondria, organelles known to have estrogen receptors (Yager and Chen, 2007). Defective mitochondria may result in compromised energy production, which could ultimately lead to anomalous meiotic spindle assembly and chromosome congression and segregation. If administration of exogenous r-FSH is associated with mtDNA defects and abnormal metaphase oocytes, current stimulation protocols should be reevaluated to reduce these detrimental effects. This possibility provides even greater support for the investment of resources into the development of successful IVM protocols, which do not incorporate exogenous stimulation.

Original research plans included incorporating IVF to investigate developmental competence of cohort oocytes. However, given the limited numbers of oocytes collected, especially from older females, and time and manpower constraints, this was not possible. Since

this aspect was intended to be included in the analyses, a study was conducted to determine the optimal cryopreservation protocol for the rhesus macaque by comparing methods previously published in several primate species. Efficient sperm cryopreservation methods would reduce the number of collections required for ART procedures, eliminate possible male effects and contribute to the well-being of semen donors. Three out of four protocols were found to be adequate for storage of rhesus spermatozoa and presumably provided spermatozoa capable of oocyte penetration and fertilization as demonstrated by ability to hyperactivate post-thaw (Boatman & Bavister 1984; Katz *et al.* 1989; Yanagimachi 1994). In situations where multiple IVF or AI cycles are scheduled during a limited time span (i.e., 4h), two methods appeared optimal if spermatozoa were incubated at room temperature post-thaw, however, the protocol developed by Seier *et al.* (1993) had the highest rates of post-thaw motility over time and was implemented as the sperm storage technique.

In summary, this study provides possible explanations for the reductions in developmental capacity of primate IVM oocytes. At present, IVM is not a suitable route for producing viable oocytes for primate ART since it may produce serious, if not lethal consequences. Clearly primate IVM conditions require improvement if IVM is to become a routine ART. It must be stressed that IVM alone will not have a large impact in conservation efforts. Reproductive science requires an integrated approach, including research on hormonal patterns and monitoring, behavior, nutrition and genetics. This study has presented baseline data on ovarian aging in the rhesus macaque and aspects of nuclear maturation during macaque IVM which may contribute to the design of primate oocyte recovery plans. This animal appears to be a faithful model for investigating problems in reproductive capability and for improving ART both in endangered primates and in human ART.

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Appendix 1.

Coauthor approval for use of the published paper:

Nichols SM, Bavister BD, Brenner CA, Didier PJ, RM Harrison, and Kubisch HM (2005)
Ovarian senescence in the rhesus monkey (*Macaca mulatta*). Hum Reprod 20:79-83.

Stephanie,

You have my permission to put the ovarian senescence paper in your thesis. Please relay this information to whomever is reviewing your thesis.

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Stephanie,

Yes, of course you may use the data from the Ovarian Senescence in Rhesus Macaque paper in your dissertation.

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As a co-author of the published chapter on Ovarian Senescence in the Rhesus Macaque, I give Stephanie Nichols approval to use this data in her dissertation. With all of my approval!!

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To whom it may concern:

As a co-author of the published chapter on Ovarian Senescence in the Rhesus Macaque, I give Stephanie Nichols approval to use this data in her dissertation.

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Appendix 2.

History of the Caribbean Primate Research Center

The CPRC is a primate research, education and training component of the Unit of Comparative Medicine (UCM) of the Medical Sciences Campus of the University of Puerto Rico. The CPRC consists of four integrated facilities: (1) Cayo Santiago, the unique free-ranging island colony of Indian rhesus macaques (*Macaca mulatta*); (2) the Sabana Seca Field Station (SSFS), the CPRC's headquarters located 10 miles outside of San Juan, Puerto Rico which houses rhesus monkeys derived from the Cayo Santiago colony in various outdoor configurations for biomedical and behavioral studies that are not feasible on free-ranging animals; (3) the Laboratory of Primate Morphology and Genetics (LPMG), located on the Medical Sciences Campus (MSC); and (4) the Laboratory of Virology and Genetics (LVG), is also located on the MSC. The locations of the CPRC facilities are shown in Figure 1.



Figure A2.1. Location of CPRC facilities. The two animal facilities are located at the Sabana Seca Field Station (SSFS) and at Cayo Santiago (CS). The Laboratories of Primate Morphology and Genetics and Virology and Genetics are located on the University of Puerto Rico Medical Sciences Campus (MSC).

The CPRC has substantial experience in the establishment and maintenance of rhesus macaque breeding colonies. Although it was nominally established in 1970, it actually dates back to 1938 with the establishment of a free-ranging colony of rhesus monkeys on Cayo Santiago (CS), an island off the southeastern coast of Puerto Rico. At that time, a John and Mary Markle Foundation grant was awarded to the research team of the School of Tropical Medicine in San Juan (predecessor of the University of Puerto Rico, School of Medicine) and Columbia University to fund an expedition to India to trap and transport Indian rhesus macaques. Over 500 rhesus macaques were trapped in the mountains near Lucknow, India by the late Dr. C.R. Carpenter. After transshipment by rail 400 miles to Calcutta, they were loaded on a steamer in September, 1938, for the 14,000 mile journey around the Cape of Good Hope to Boston and New York City. The monkeys eventually arrived in San Juan in December, 1938 and were released onto Cayo Santiago (Figure 2).



Figure A2.2. Birdseye and boat view of Cayo Santiago. The island is located 0.5 miles off the southeastern coast of Puerto Rico.

Cayo Santiago is the oldest continuously maintained colony of its kind in the world. The CS colony of Indian-origin rhesus monkeys has been closed since 1940 and its derivative colony at the SSFS has been closed since the mid-1980s. Replacement stock at both facilities is recruited only through births. Many hundreds of monkeys have been systematically removed

from the colony over the years to supply research animals for many institutions, providing an invaluable service to the national and international scientific community by offering Indian-origin rhesus macaques with known backgrounds and of the same genetic pool.

Sabana Seca Field Station: The SSFS houses monkeys that are derived from the Cayo Santiago colony or their offspring born at SSFS (Figure 3). It is also the administrative headquarters and base of operations for the animal care program of the CPRC and location of the experimental embryology laboratory. The Sabana Seca facility (Figure 3) is located approximately 10 miles west of San Juan on a 252-acre tract of land, the vast majority of which is undeveloped hills, forest or wetlands that serve as a buffer to noise. This provides the monkeys with a relatively quiet and naturalistic sound environment. There are currently over 1100 rhesus monkeys occupying the 176,000 ft² of animal housing facilities, including both conventional colony (consisting of virus-positive animals, e.g., Herpes-B virus, a naturally occurring virus in macaques) and specific pathogen free individuals. Study animals for the experimental embryology program are taken from the conventional colony.



Figure A 2.3. Aerial view of the Sabana Seca Field Station.

Vita

Stephanie Michelle Nichols was born in Abington, Pennsylvania where she attended Catholic schools for 12 years. She went to the College of William and Mary in Williamsburg, Virginia for her undergraduate education and received a B.S. in Biology. Stephanie then attended Moss Landing Marine Laboratories in Moss Landing, California as a part of the San Jose State University's graduate program in Marine Science. Her formal education in the marine sciences took place in California, while her research was conducted at the Mote Marine Laboratories Center for Shark Research in Sarasota, Florida. Ms. Nichols' research focused on the potential role of the hormone calcitonin in the reproduction and development of the bonnethead shark, *Sphyrna tiburo*. She received her M.S. in Marine Science in 2001. In 2002, Stephanie entered the Conservation Biology Program at the University of New Orleans in New Orleans, Louisiana with an interest in the application of reproductive technologies in species conservation. During her graduate experience as a doctoral student, she had the opportunity to collaborate with several institutions specializing in primate assisted reproductive technologies including: the Kunming Institute of Zoology in Kunming, China; Tulane National Primate Research Center in Covington, Louisiana; and the Caribbean Primate Research Center in Sabana Seca, Puerto Rico. In 2005, Stephanie relocated from New Orleans to Puerto Rico to collaborate with the Caribbean Primate Research Center in the establishment of an embryology laboratory and to continue her dissertation work. Ms. Nichols completed her dissertation research and received her Ph.D. in Conservation Biology in May 2007.