VARIABLES INVOLVED IN GAMETE RECOVERY, IN VITRO FERTILIZATION, EMBRYO CULTURE AND TRANSFER IN THE SQUIRREL MONKEY (SAIMIRI SCIUREUS)

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Thomas John Kuehl

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W Richard Dukelow
Major professor

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ABSTRACT

VARIABLES INVOLVED IN GAMETE RECOVERY, *IN VITRO* FERTILIZATION, EMBRYO CULTURE AND TRANSFER IN THE SQUIRREL MONKEY *(SAIMIRI SCIUREUS)*

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These studies were conducted to examine the procedures and variables of an *in vitro* fertilization system used to provide zygotes for transfer to recipient females. A laparoscopic technique was developed to place a fertilized zygote (in 1 to 3 µl of fluid) into the ampulla of the oviduct. By use of the laparoscope and a micropipette, transfer can be performed to the oviduct *in situ* without the trauma of laparotomy.

Computer programming techniques were used to aid in the collection, compilation, and analysis of data from *in vitro* fertilization experiments. A program was designed for interactive use by the investigator and can produce historical data on individual animals, such as seasonal weight changes of male squirrel monkeys with associated changes in reproductive variables (ejaculate volume and sperm production). These techniques were expanded to a Macaque reproduction data bank and modeling of plasma luteinizing hormone levels in the rhesus monkeys.

In the course of *in vitro* fertilization trials, 745 oocytes were aspirated from 2,168 follicles (34.4% recovery rate). Of these oocytes,

137 (18.4%) were atretic. Thirty-eight percent of the remaining 608 oocytes matured to the metaphase II stage. Differences in aspiration procedures did not affect maturation or *in vitro* fertility. Male squirrel monkeys used to supply semen were of significantly different fertility. Volumes and cellular composition of follicular fluid were determined for four sizes of follicles. Small (1 mm diameter) follicles yielded significantly higher proportions of atretic ova. Five percent CO_2 in air and a medium containing both pyruvate and serum proteins yielded the highest fertilization rates. Addition of either 10 or 100 m.i.u. of insulin to this culture medium did not alter *in vitro* maturation or fertilization, but did significantly reduce the formation of monolayers by follicle cells.

Thirteen embryo transfers were performed, but no confirmed pregnancies were found.

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Love is the light
that leads the way
as we walk
the path of life.

--Bonnie Kuehl

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To the greatest love of my life, only love can express my thanks and appreciation. Its almost time.

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INTRODUCTION

In vitro fertilization of human ova and subsequent transfer of the human blastocyst into the uterus of a woman, either the ova donor or a foster mother, are coming soon. This procedure may even become clinically routine in the near future. Yet, there is considerable controversy surrounding this area of research. With the current interest in birth defect studies, the possibility of screening cultured embryos before they are reimplanted becomes both terrific and terrifying. Before these procedures arrive, studies into their genetic safety are desirable. Most of the current primate research in this area of reproductive physiology involves human material. Suitable nonhuman primate models have not been developed.

Recently *Saimiri sciureus* ova have been fertilized successfully *in vitro*. Techniques are available to develop this species as such a model. The variables involved in obtaining fertilized ova need to be examined with the goal of providing early preimplantation embryos to test the genetic safety of embryo transfer. Because squirrel monkeys are expensive to maintain, procedures must be developed which maximize their use by recording as much data as possible. With the advent of the computer in biomedical research, a powerful tool exists to manage vast amounts of data. Computer routines can be used to manage large data banks for use by investigators in determining basic reproduction values. The objectives of these investigations were:

1. To develop a laparoscopic procedure for placement of fertilized ova into recipient oviducts,
2. To develop and employ computer routines to manage and analyze data on reproductive phenomena,
3. To further refine procedures and identify important variables for production of *in vitro* fertilized *S. sciureus* ova,
4. To transfer fertilized ova to recipient animals.

VARIABLES OF *IN VITRO* FERTILIZATION AND TRANSFER SYSTEMS IN *SAIMIRI SCIUREUS*

Literature Review

In Vitro Fertilization in Primates

Several reviews have been published in recent years documenting the progress of research on oocyte maturation and fertilization *in vitro* which lead to successful work on embryo transfer (Chang, 1968; Thibault, 1969; Austin, 1970; Brackett, 1970; Kennedy, 1972; Edwards and Steptoe, 1975). In light of these publications, the present review will be limited to accounts of *in vitro* fertilization in primates.

The history of studies in primate *in vitro* fertilization can be divided into three periods: (1) prior to 1951; (2) from 1951 to 1969; and (3) from 1969 to the present. The first period was marked by studies such as the report of *in vitro* fertilization of guinea pig and rabbit uterian oocytes (Onanoff, 1893). Following the discovery of capacitation in 1951, a wealth of basic studies were performed. *In vitro* fertilization was successfully accomplished in a variety of species, but until 1969 few studies were recorded for primate species. In 1969, the present period of *in vitro* fertilization was entered with the first definitive *in vitro* fertilization of human eggs (Edwards, Bavister and Steptoe, 1969).

The first period consists of early accounts of *in vitro* fertilization which were cast in doubt with the discovery of sperm

capacitation by Austin (1951) and Chang (1951). During this period human *in vitro* fertilization was first reported by Menkin and Rock (1944, 1948). They observed over 800 oocytes and attempted to fertilize 138. After culturing the oocytes with washed spermatozoa, they obtained 2 two-celled and 2 three-celled embryos. Doubt was cast on these studies as one of the three-celled embryos appeared grossly abnormal and no observation of polar body formation or signs of maturation were reported.

The second period in the history of studies of primate *in vitro* fertilization consists of reports of *in vitro* fertilization of human ova which have been cast in doubt due to inconsistent methodology, lack of adequate evidence of fertilization, the possibility of parthenogenesis and abnormalities of maturation or fertilization. Early in this period, Shettles (1953, 1955) studied over 1,000 oocytes, but only 200 were subjected to insemination. They were cultured with fresh human sperm in a medium of follicular fluid with bits of tubal mucosa added for capacitation. Six oocytes were reported to have undergone cleavage, one to a 32-celled morula by 72 hours post-insemination. Lack of observation of intermediate stages of development of the morula and signs of maturation of the oocytes cultured have placed some doubt on these studies. Petrucci (1961) reported human *in vitro* fertilization, but signs of maturation and incidence of either successful culture, fertilization or parthenogenesis were not given. This work has never been published in an acknowledged scientific journal. Hayashi (1963) observed fertilization in 20 of 160 follicular oocytes he cultured

in vitro in media with human serum proteins, basal salt solution and hormones. The oocytes were cultured with cumulus cells left intact around them. Although penetration of spermatozoa, formation of pronuclei and cleavage to the morula stage were observed, Hayashi stated cleavage to be morphologically indistinguishable from parthenogenesis.

Near the end of this period, investigators began studying *in vitro* maturation of primate oocytes in order to provide more mature ova for fertilization from the limited number recovered. Several investigators reported *in vitro* maturation of human oocytes (Edwards, 1965; Kennedy and Donahue, 1969) in either defined (F-10 with 4 mg/ml bovine serum albumin) or undefined (TC-199 with 15 to 20% fetal calf serum) media to the metaphase I or II stage. In 1968, Jagiello, Karnicki and Ryan collected oocytes from women pretreated with gonadotropins (pituitary follicle stimulating hormone (FSH) and human chorionic gonadotropin (HCG)). They found that by 22 hours after HCG administration a third of the oocytes were at metaphase I. Of oocytes obtained in this manner and cultured for an additional 25 to 28 hours, all resumed meiosis and two-thirds reached metaphase II. Suzuki and Mastroianni (1968) examined the fertility of *in vitro* matured nonhuman primate oocytes. They matured oocytes from *Macaca mulatta* in Waymouth medium with 10% rhesus monkey serum and then transferred them to oviducts of inseminated females. Fifteen of 31 oocytes, which had been matured, transferred, and recovered 24 to 48 hours later, were definitely not fertilized. Eight ova were degenerated, six contained

two polar bodies or two pronuclei and two contained two degenerating blastomeres each. The authors suggested that a failure of cytoplasmic maturation *in vitro* may have contributed to reduced fertility of ova matured *in vitro*. In 1969 the modern period of studies of primate *in vitro* fertilization was entered as Edwards, Bavister and Steptoe (1969) reported uncontestable fertilization *in vitro* of human oocytes matured *in vitro*. Their technique involved culturing follicular oocytes 35 hours in a variety of media containing follicular fluid, inseminating the cultures with washed spermatozoa and monitoring for pronuclei formation. No pronuclei were seen in control ova, but 11 pronucleate eggs were found in inseminated cultures. Some ova were reported to have as many as five pronuclei suggesting polyspermy. They indicated that extra bicarbonate and follicular fluid were added resulting in a higher pH (7.6) than that previously used for *in vitro* fertilization of human ova. Fertilization was further verified by identification of the midpiece and tail of the fertilizing sperm in the ooplasm of these eggs (Bavister, Edwards and Steptoe, 1969).

The onset of the modern period was characterized by the development of several other successful systems for *in vitro* fertilization of human ova. In 1969 Jacobson, Sites, and Arias-Bernal reported such a system for *in vitro* maturation and fertilization of human follicular oocytes. They cultured oocytes in a medium of Hank's solution, 20% fetal calf serum and 5% human follicular fluid. Forty-one oocytes were cultured singly in microdrops, some with about 50 motile sperm. More than 50% of the ova inseminated cleaved and most

were found to contain the normal diploid chromosome number. Eight of 11 were female embryos (46XX) and three were male embryos (46XY). All had cleaved to at least the four-celled stage.

In 1970 Steptoe and Edwards were able to recover *in vivo* matured oocytes by laparoscopy from preovulatory follicles of women treated with human menopausal gonadotropin (HMG). Based on this study HMG and HCG were used to stimulate *in vivo* maturation of human oocytes (Edwards, Steptoe and Purdy, 1970). Laparoscopies were performed 30 to 32 hours after the final HCG injection and 393 oocytes were obtained for culture. The oocytes were cultured 1 to 4 hours, inseminated and and examined for evidence of fertilization at varying intervals. Several media were tried but Ham's F-10 medium modified to pH 7.6 gave the best results. Forty-five of the pronucleate ova that resulted from insemination went on to cleave. These investigators speculated that follicular fluid or granulosa cells were important for fertilization. Steroids in the follicular fluid or synthesized by granulosa cells were thought to affect the sperm acrosome and provide for capacitation *in vitro*. Seitz et al. (1971) went a step further by adding sodium estrone sulfate (Premarin) to their medium of Ham's F-10 and heat-inactivated human serum. Their system used human oocytes matured *in vitro*. Ova were matured for 24 hours in culture, then inseminated with either washed spermatozoa or sperm which were preincubated in a rhesus monkey uterus. Eight of 50 ova in culture with preincubated spermatozoa were fertilized and ranged in development from the two-celled stage at 40 hours to a twelve-celled stage by 72 hours after

insemination. None of 15 ova exposed to washed spermatozoa cleaved, but through use of time lapse cinematography, the authors observed penetration of the zona pellucida. They concluded that if capacitation were required for penetration of the human zona pellucida, it could be accomplished *in vitro* without exposure to fluids from the female tract.

In 1972 studies of primate *in vitro* fertilization were expanded to nonhuman primate species. Following the model of Seitz et al., investigators added estrone to their culture medium in an attempt to *in vitro* fertilize squirrel monkey oocytes (Johnson et al., 1972; Johnson, 1972). They reported that spermatozoa attached to the zona pellucida of follicular oocytes cultured in tissue culture medium (TC-199) plus 25 μg/ml estrone sulfate with either 20% heat-inactivated squirrel monkey serum or agamma fetal calf serum. Of four oocytes cultured with spermatozoa, one showed the sperm attachment and two had sperm in their surrounding cumulus cell masses. In another study, Cline et al. (1972) reported fertilization *in vitro* of *in vivo* matured *S. sciureus* oocytes. They used a medium of TC-199 with 20% heat-inactivated agamma newborn calf serum plus bovine serum albumin. Of 16 mature oocytes recovered from squirrel monkeys pretreated with pregnant mare's serum (PMS), 7 appeared fertile, 5 of which developed to the two-celled stage. In an expanded study, 22 mature ova were utilized in an *in vitro* fertilization system employing preincubation of ejaculated spermatozoa in follicular contents plus medium prior to insemination of test cultures (Gould et al., 1973). Eleven of the 22 ova showed sperm in the perivitelline space, extrusion of the second polar

body or pronuclei formation. Six ova went on to cleave to the two-celled stage. Twenty-four oocytes matured *in vitro* and cultured in this system showed no signs of fertilization, further suggesting a reduced fertility of ova *in vitro* matured.

Kraemer (1973) described *in vitro* systems for maturation and fertilization of oocytes in yet a third primate, the baboon *(Papio cynocephalus)*. Baboon follicular oocytes were cultured in a variety of media including TC-199 with 15% calf serum, TC-199 with baboon serum, follicular fluid, Brinster's medium and 50% follicular fluid with Brinster's medium. The highest percentage of maturation to metaphase II after 48 hours of culture was obtained with 50% follicular fluid-50% Brinster's medium but no attempt was made to fertilize these oocytes. Instead, follicular oocytes recovered from the ovaries of baboons primed with gonadotropins were cultured in a variety of fertilizing media including follicular fluid, Brinster's medium and Bavister's medium. They received sperm which were either fresh, incubated in the uterus or in the cervical mucus. Incubation with sperm lasted 8 hours followed by transfer of the ova to Ham's F-10 with 20% heat-inactivated calf serum for 72 hours. Most of the oocytes were still in the germinal vesicle stage after 72 hours of culture. Four oocytes were fertilized in Brinster's and Bavister's media and were found to have two polar bodies and two pronuclei. One six-celled ovum was recovered after fertilization in Bavister's medium by sperm which had been incubated in cervical mucus. All six nuclei were visible. No other primate species have been successfully *in vitro* fertilized to date.

Recently some investigators of primate *in vitro* fertilization have more closely examined the *in vitro* maturation of follicular oocytes and the capacitation requirement of primate sperm. Dukelow and Chernoff (1969) found indirect evidence for a need of capacitation by human and rhesus monkey sperm. This requirement could be at least partially accomplished in a foreign uterine environment (the hamster uterus being most suitable. Soupart has examined both *in vitro* maturation and sperm capacitation in the development of a system for *in vitro* fertilization of human oocytes. Soupart and Morgenstern (1973) obtained *in vitro* penetration by sperm through the zona pellucida and fertilization of oocytes that were recovered from ovaries of gonadotropin-treated women. They used a two-media culture system. The oocytes were first cultured for 38 to 48 hours in a maturation medium containing 75% follicular fluid and 25% modified Bavister's medium (used successfully by Edwards et al., 1970) with or without exogenous gonadotropic hormones (FSH, LH, and HCG). Control ova were cultured in the same fertilization medium without exogenous hormones. The results showed no effect of exogenous gonadotropins in the maturation culture on the proportion of oocytes maturing. Overall, 34% of the oocytes matured to the metaphase II stage. The authors found that gonadotropins in the fertilization media did significantly increase sperm penetration into or through the zona pellucida. More oocytes showed sperm penetration than matured, leading the authors to conclude that sperm penetration is not dependent on oocyte maturation but may require sperm capacitation. The enhancement of sperm penetration was thought to be mediated by the gonadotropin

stimulation of the follicle cells present among the oocytes in the fertilization medium. The authors also observed that the tail of the fertilizing sperm or its remnants could not be found in oocytes recovered 28 hours after insemination, either in the fresh state or after fixing and staining. Bavister et al. (1969) had previously reported observing the midpiece and tail of spermatozoa in human fertilized ova 11 to 14.5 hours after insemination of oocyte cultures with washed sperm. Soupart and Strong (1974) later found bits of degenerating sperm tail in oocytes of the late pronucleate stage, but these were only visible by means of electron microscopy. They also found microscopic evidence that sperm cells passing through the zona pellucida had undergone the acrosomal reaction which may have taken place during contact with follicular cells in the culture medium. Soupart and Strong did not observe sperm penetration through the zona until 5 hours after sperm were exposed to ova and follicular cells in the culture. They pointed out that this delay was consistent with other investigator's reports of not observing sperm presence in the perivitelline space until 7 to 7.5 hours after insemination (Edwards et al., 1970). The authors concluded that 5 to 7 hours are required for human sperm capacitation in the *in vitro* fertilization environment.

In his most recent paper, Soupart (1975) described a two-stage maturation culture system for immature human oocytes. He hypothesized that immature oocytes transplanted into a culture medium, enter an environment in which their satellite cells (corona radiata and cumulus oophorus) are much less numerous than in their original follicles. To

remedy a possible lack of estrogen and progesterone production which might normally act in regulation of maturation, the oocytes were cultured sequentially in Ham's F-10 medium containing these steroids. First, oocytes were primed in culture with estradiol-17β (0.1 μg/ml) for 4 hours. Then the oocytes were incubated in the presence of 17α-hydroxyprogesterone (0.1 μg/ml) for 40 to 44 hours. At the end of the 44 to 48 hours of culture, 45% of the immature oocytes were in the metaphase II stage. This system may not yet be optimal for *in vitro* maturation of human oocytes, as Jagiello et al. (1975) have found maturation of human oocytes *in vitro* can be stimulated by ovine LH, estradiol-17β (with LH added later), and prostaglandin E_1. They also examined rhesus monkey oocytes and found stimulation of *in vitro* maturation by ovine LH and prostaglandin E_1. However, the fertility of these oocytes (Jagiello et al., 1975) was not examined. Soupart was able to achieve 20% fertilization *in vitro* of oocytes matured by the two-stage method.

At the present, these studies seem to indicate that both *in vivo* and *in vitro* matured primate oocytes can be fertilized *in vitro*. Contents of ovarian follicles, such as follicular fluid and cumulus cells, which are aspirated along with the oocytes seem to play a role in the capacitation process *in vitro*. Other variables, such as hydrogen ion concentration, oxygen tension, osmotic pressure, and composition of the culture media (including additions), are probably important to the success of a given *in vitro* fertilization system. These will be described in the discussion of the results of the present research.

The modern period of primate *in vitro* fertilization with its predominate human orientation, has brought with it the possibility of providing embryos by methods other than *in situ* fertilization for gestation in infertile women. The relative lack of nonhuman primate models to study this process (and especially the teratogenic potential) first led the author to develop such a system with the squirrel monkey (Kuehl, 1974; Kuehl and Dukelow, 1975). The examination of the variables in this system following the initial development is one subject of this work.

Embryo Transfer in Primates

Several reviews of embryo transfer as well as a discussion of transfer techniques will be provided in a later portion of this dissertation (page 49). This section will be limited to discussion of the specific application of embryo transfer techniques to primates. To date, there are no scientific reports where a primate (human or nonhuman) embryo has been successfully transferred to yield a pregnancy. There have been three published accounts of successful embryo transfer in the popular press. The first was a Wire Services' story of July 17, 1974, quoting Dr. Douglas Bevis of the University of Leeds, England, as stating that 3 of 30 embryo transfers attempted in women had resulted in the birth of live, normal young. The details and subsequent scientific publication of this study have not been forthcoming. In another account, Dr. P. C. Steptoe described an ectopic pregnancy following transfer of a human blastocyst to the uterus. The blastocyst was grown in culture from an *in vitro* fertilized human oocyte. Finally, Dr. D. C.

Kraemer (Personal communication, 1975) has transferred an *in vivo* fertilized baboon embryo, by surgical technique, to the uterus of a recipient baboon. A live, normal infant was born at term following this transfer and work is currently underway to verify the parentage. Other than these three accounts, a primate uterus has yet to implant an embryo placed there artificially.

Three factors can be differentiated which may affect the outcome of an embryo transfer. These are (1) the stage of development of the transferred embryo, (2) the route of transfer, and (3) the implantation and fetal growth after transfer. All three factors must be considered in getting the birth of a live, healthy infant.

Ova and early embryos in various stages of development have been successfully transferred in a number of nonprimate species (reviewed by Chang and Pickford, 1969; Dzuik, 1969; Adams and Abbott, 1971; Edwards and Steptoe, 1975). The particular stage of development was not important to success as long as synchrony between the stage of development of the embryo and the endocrine status of the recipient was attained. Table 1 compares the early embryonic development of three species of nonhuman primates and man. While implantation varies from 7 days after ovulation in the human (Hertig, 1975) to 9 days in the rhesus monkey (Heuser and Streeter, 1941) and baboon (Hendrickx and Houston, 1971), the time course of early cell division in these species is the same. Recently, Eddy et al. (1975) have shown the oviductal transport time of rhesus monkey ova to be 72±12 hours. Thus as the fertilized zygote reaches the uterus, it is in the eight-celled stage,

Table 1. Time course of early embryonic development in primates (hours)

Species	Second polar body	Cleavage divisions				Morula	Blastocyst
		First	Second	Third	Fourth		
Saimiri sciureus							
*in vivo**							(96 to 120)[a]
Macaca mulatta							
in vivo		29.5[b]			(96)[b]		
*in vitro***		24 to 36[b]	36 to 48[b]	48 to 72[b]	72 to 96[b]		
Papio cyncephalus							
in vivo	24[c]		48[c]	(48 to 72)[c]	(96 to 120)[c]	(120 to 148)[c]	(< 120)[c]
Homo sapiens							
in vivo		30[f]		72[g]	(96)[df]	(96 to 120)[d]	(< 120)[df]
in vitro	11.5[e]	< 38[e]	38 to 46[e]	51 to 62[e]	< 85[e]	111 to 135[e]	123 to 147[e]

*Entries for *in vivo* indicate the time in hours after ovulation the embryonic stage was flushed from oviduct. Parentheses around entry indicate embryo flushed from uterus.

**Entires for *in vitro* indicate the time in hours after insemination the embryonic stage was observed in culture.

[a]Ariga, 1976. [b]Lewis and Hartman, 1933. [c]Kraemer and Hendrickx, 1971.

[d]Croxatto et al., 1972. [e]Edwards and Steptoe, 1975. [f]Hertig et al., 1954.

[g]Avendano et al., 1975.

which is in agreement with the *in vivo* observations shown in Table 1. To be in synchrony, primate transfers of two-polar-body, two-celled and four-celled zygotes should be made to the oviduct of the recipient by 24, 36, and 48 hours, respectively, after ovulation. Embryos of eight or more cells should be transferred to the uterus at or slightly before the time they would normally be found *in vivo*. Transferring a given stage of embryo to the appropriate site and at the proper time following ovulation, will ensure synchrony of the embryo to the endocrine state of the recipient.

The route of transfer refers to the technique used in depositing an embryo in the female reproductive tract. Both surgical and non-surgical techniques have been used in laboratory and domestic animals (see discussion, page 49). In baboons, Kraemer (1975) used a surgical procedure to expose the uterus. Passage through the uterine wall to the lumen was done by needle puncture. A fine catheter was passed through the needle to the uterine lumen. Embryos of more than eight cells were flushed into the lumen in a small volume of medium. Edwards and Steptoe (1975) preferred the cervical approach in women. Their method required only local anesthesia and no puncture of the uterine wall. A catheter was passed directly through the cervix and a blastocyst was injected into the uterine lumen. No oviductal transfers have been described in primates. The important consideration with regard to route of transfer is to select the pathway resulting in the least damage to both embryo and the recipient.

A final consideration for successful embryo transfer is implantation and development of a normal pregnancy. If the embryo has been transferred in synchrony with the recipient and the route of transfer has not been damaging, a pregnancy should result. Failure of a pregnancy in primates may be due to embryonic death. Recently, Edwards and Steptoe (1975) have described a phenomenon in women which is of particular importance to primate systems of embryo transfer. They reported that histological examination of the uteri of women treated with gonadotropins for the recovery of oocytes revealed the endometrium to be secretory. They suggested that this uterine stimulation may be inadequate for implantation because the interval between administration of HCG and menstruation was shorter in treated women than the interval between the LH surge and menstruation in the normal cycle. They suggested that the recipients, which receive gonadotropins for stimulation of follicular growth, be given additional steroid hormones or HCG (to support the corpora lutea) for two weeks following transfer. This procedure might prevent failure of the pregnancy due to a luteal deficiency. This consideration is important as primate recipients are often primed with gonadotropin to provide *in vivo* matured ova or to synchronize them with the embryo to be transferred

Materials and Methods

Recovery of Gametes from Experimental Animals

Both male (7) and female (96) adult squirrel monkeys *(S. sciureus)* of either Brazilian (Tarpon Zoo, Inc., Tarpon Springs,

Florida) or Bolivian (Primate Imports Corp., Port Washington, New York) type were used in these studies. The maintenance of these animals has been previously described (Kuehl and Dukelow, 1975). In brief, animals were housed in groups of two to five in stainless steel, flush type cages or in a large group (30 to 50) in a colony cage (Harrison, 1973). Fresh water was supplied *ad libidum*. The diet consisted of commercial monkey feed (Wayne Monkey Diet, Allied Mills, Chicago, Illinois). The animal quarters had controlled fluorescent lighting on a 12:12 light-dark cycle and was temperature-controlled at 21±2°C. Relative humidity ranged from 40 to 60% dependent on season.

Oocytes were recovered from gonadotropin treated females just prior to the time of expected ovulation. Female squirrel monkeys were induced to ovulate without regard to their natural cycle using a regime previously reported (Dukelow, 1970) with modifications during the anovulatory season (Kuehl and Dukelow, 1975). The regime consisted of four daily intramuscular injections of follicle stimulating hormone (FSH) (1 mg, FSH-P, Armour-Baldwin Laboratories, Omaha, Nebraska) with an intramuscular injection of 500 iu of human chorionic gonadotropin (HCG) (A.P.L., Ayerst Laboratories, New York, New York) on the morning of the fifth day. From June 15 to October 15 of each year (anovulatory season) a fifth FSH injection was added. Neither progesterone nor estrogen injections were used in this regime. Oocytes were recovered by aspiration from the follicles. Two aspiration procedures were used. The first was a surgical procedure, laparotomy, used to expose the ovaries for aspiration using a 1 ml tuberculin syringe containing

0.05 to 0.10 ml of culture medium. As described previously (Kuehl and Dukelow, 1975), all visible follicles were punctured laterally with a 25 gauge needle (bevel side down) and the contents drawn into the syringe. The second procedure used the laparoscope to observe the ovaries (Dukelow et al., 1971). Follicles observed in this way were measured and classified according to stage of development. Aspiration was as stated above, except that the needle was inserted through the skin and abdominal wall to reach the ovaries. Follicles were aspirated as early as 4 hours and as late as 56 hours after HCG administration but usually 8 to 12 hours after HCG.

Spermatozoa were recovered from male squirrel monkeys by electroejaculation using the method and restraint system described by Kuehl and Dukelow (1974). The ejaculate, in the form of coagulum, was incubated in 1 to 2 ml of culture medium at either room temperature (23°C) or 37°C for 0.2 to 2 hours. The resulting sperm suspension was aspirated from the sample tube containing the coagulum and used to inseminate oocyte cultures.

Culture Environment

The culture environment consisted of four components: (1) culture vessel, (2) temperature, (3) atmosphere, and (4) medium. One culture vessel, a chamber-slide (tissue culture chamber/slide, No. 4808, Lab-Tek Products, Naperville, Illinois) with eight chambers, was used throughout the course of these studies. The incubation temperature was maintained at 37 to 38°C. The area used for gamete collection and culture examination was maintained at or above 30°C

during all procedures. Five culture atmospheres were examined. All test atmospheres contained 5% carbon dioxide, with variable proportions of moist oxygen (0, 10, 20, 30, and 95%). Nitrogen was used to complete the balance. Based on these results, an atmosphere of 5% carbon dioxide in air (20% oxygen) was used in all the other studies. Five different media were tested in the culture system. Table 2 compares the composition of these media. Gametes were incubated in only one medium throughout a trial period.

Transfer of Fertilized Ova to Experimental Animals

Mature oocytes, fertilized *in vitro*, were transferred to the oviducts of recipients using the procedure described on page 51. The recipients were synchronized with the embryo donors by the ovulation induction procedure. All transferred embryos (2 polar body--2 pronucleate to four-celled zygotes) were deposited in the oviduct of the recipient ipsilateral to the ovary with a corpus luteum. Following the transfer, some recipients received either HCG (50 iu) or progesterone (5 mg, Δ 4-pregnen-3, 20-dione, Sigma Chemical Company, St. Louis, Missouri). All recipients were dianosed for pregnancy using the procedures discussed in Appendix A.

Experimental Design

Variables of the *in vitro* fertilization system were examined by conducting multiple trials. A trial consisted of the recovery of male and female gametes, the incubation period with several samples, and a transfer procedure when ova were fertilized and a recipient

Table 2. Composition of *in vitro* fertilization culture media

Ingredient	Media composition				
	TJK-1	TJK-2	TJK-3	TJK-4	TJK-5
TC-199*[a]		80%	80%	100%	100%
Ham's F-10*[b]	80%				
Serum*[c]	20%	20%	20%		
BSA**[d]				6 mg	6 mg
Pyruvate**[e]			72 µg	72 µg	72 µg
Penicillin**[f]	50 units	50 units		50 units	
Pen-strep**[g]			100 units		100 units
Heparin**[h]	1 unit	1 unit	1 unit	1 unit	1 unit

*Percent of total volume.

**Units per ml.

[a]Medium 199 with Earle's salts and L-glutamine, North American Biologicals, Inc., Rockville, Maryland.

[b]Ham's F-10 with L-glutamine, North American Biologicals, Inc., Rockville, Maryland.

[c]Agamma newborn calf serum, North American Biologicals, Inc., Rockville, Maryland.

[d]Crystallized albumin from bovine serum, Sigma Chemical Co., St. Louis, Missouri.

[e]Sodium pyruvate, Sigma Chemical Co., St. Louis, Missouri.

[f]Potassium penicillin G, Calbiochem, San Diego, California.

[g]Penicillin-dyhydrostreptomycin mixture, North American Biologicals, Inc., Rockville, Maryland.

[h]Sodium heparin, Wolins Pharmacal Corp., Melville, New York.

female was available. Variables were altered one at a time over the course of several trials. A series of trials was conducted for each variable with all the values of the variable to be compared. The series of oxygen tensions, for example, had five trials per series. Within a trial, treatments were examined, with each treatment occupying a separate chamber. Chambers with medium alone, medium plus spermatozoa suspension, and medium plus follicular aspirate (no sperm) served as controls for pH measurements, sperm motility and fibroblasting of cumulus cells noted during an incubation period. During the course of these studies, other investigators suggested that addition of insulin to the culture medium would improve oocyte maturation and fertilization. The three levels tested (0, 10, and 100 m.i.u./ml, crystalline bovine pancreatic insulin, Sigma Chemical Co., St. Louis, Missouri) were added to chambers within a trial in 5 μl of 0.15 molar sodium chloride, thus constituting three treatments within a trial. The females used for trials were randomly assigned and treated with an equivalent ovulatory regime of gonadotropins throughout the year to minimize seasonal effects. Males were not controlled for seasonality, but sperm concentrations were maintained at $2.0 \pm 1.0 \times 10^6$ spermatozoa per ml for the 0.5 ml culture volume. Effects due to male differences in fertility were minimized by using sperm from only one male per series of trials. Treatments within a trial, of course, received the same sperm suspension.

The data from each trial was placed on computer file (see discussion of DAS system, page 61) by experimental unit. For example,

an appropriate data form was completed for each male, female, and culture chamber. Listings were compiled from this data bank. As maturation, fertilization, and fibroblasting of cumulus cells are dichotomous measurements, Chi-square with Yates' correction for discrete data was used to analyze the results (Spiegel, 1961). Culture effects on maturation were isolated from other effects by examination of the number of ova maturing *in vitro*. Stage of development of an ovum was determined before and during *in vitro* culture by microscopic observation of the chamber slide. At the end of the culture period (50 to 80 hours), the stage of development was confirmed by staining with lacmoid (Iwamatsu and Chang, 1972). For ova to be selected for transfer, the following criteria for fertilization were used:

1. Two or more polar bodies present in the perivitelline space and two pronuclei within the ooplasm.
2. Two or more polar bodies present in the perivitelline space; two equal sized blastomeres.
3. Two or more polar bodies present in the perivitelline space; four equal sized blastomeres.
4. Five or more blastomeres of sizes that could be associated with cleavage type divisions.

Criteria two through four had to be verified by several observations to ensure that fragmented ova were not mislabeled as multicelled embryos. Where a culture chamber contained more than one oocyte, the relative positions of each of the oocytes were noted. Thus an individual oocyte could be followed throughout the culture period.

Results

A total of 745 oocytes were recovered from 2,168 follicles for a 34.4% recovery rate. Of these oocytes 137 (18.4%) were classified as atretic. These oocytes showed either darkening, vesicularization and shrinkage of the ooplasm or uneven division of the ooplasm (fragmentation) when first observed in culture. They were not considered capable of maturation, leaving 608 oocytes with the possibility of reaching the metaphase II stage. Thirty-eight percent did, and 78 of these were fertilized *in vitro* by spermatozoa from one of the five males listed in Table 3. There was a significant ($p < 0.05$) difference in the fertility of the males. Of the 46 mature ova not exposed to sperm, none showed any nuclear formations suggestive of parthenogenesis and none cleaved. A comparison of the techniques of ovum recovery is shown in Table 4. No significant differences ($p < 0.05$) were found for any of the comparisons between laparoscopy and laparotomy.

While there were no differences in oocyte recovery, atresia, and maturation between aspiration techniques, considerable differences were found among groups of ova from follicles of different sizes. These results are shown in Table 5. There was a significant ($p < 0.05$) difference in the recovery rates from the various follicles, with 1 mm diameter follicles yielding a significantly higher proportion of atretic ova. The fertility of oocytes recovered from the various sized follicles was similar except for those from the 1 mm diameter follicles. There was also a tendency for larger follicles to yield oocytes that would mature to the metaphase I stage or beyond in culture. Other

Table 3. Fertility of males used as sperm donors

Male	Number of ejaculates used	Number of mature ova inseminated	Number fertilized*	Percentage
DUKE	41	100	40	40.0
MICK	11	36	24	66.7
1399	7	13	1	7.7
1400	11	34	12	33.3
HIRO	1	4	1	25.0
Total	71	187	78	41.7

*Significant difference ($p < 0.05$) when analyzed by χ^2 method for male.

Table 4. Comparison of follicle aspiration techniques

Technique	No. of follicle aspirated	Oocyte recovery		Atretic oocytes		Matured ova	
		No.	% of follicles	No.	% of oocytes	No.	% of nonatretic oocytes
Laparoscopy	708	249	35.2	39	15.7	91	43.3
Laparotomy	1,460	496	34.0	98	19.8	142	35.7
Total	2,168	745	34.4	137	18.4	233	38.3

Table 5. Comparison of recovery, maturation, and fertilization of ova from follicles of varying diameter

Follicular diameter (mm)	Oocyte recovery*		Atretic oocytes		Maturing ova[a]		Fertile ova[b]	
	Oocytes/ follicles	% of fol. asp.	No.	% of oocytes	No.	% of non-atretic	Fertile/ matured	% of matured
Preovulatory (3-4 mm with dome)	18/56	32.1	2	11.1	12	75.0	4/7	57.1
3	152/369	41.2	20	13.2	78	59.1	23/56	40.4
2	137/371	36.9	24	17.5	66	58.4	18/36	50.0
1	79/266	29.7	26	32.9**	26	49.1	3/18	16.7

[a]Includes both maturing and matured.

[b]Percent of matured includes only matured ova exposed to sperm (19 ot 137 matured served as non-inseminated controls).

*Significant difference ($p < 0.05$) when analyzed by χ^2 method for follicle size.

**Significantly different ($p < 0.05$) from same measure for other follicle sizes.

differences between these follicles are considered in Tables 6 and 7. Only data from successful aspirations (yielding ova) were included in these tables. Other than follicle surface diameter, the actual physical appearance of the oocyte can be used for classification.

Soupart and Morgenstern (1973) postulated the classification system shown in Table 8, which groups oocytes by the appearance of their cellular investments. Three mature ova were classed as atretic, however lacmoid staining revealed metaphase II chromatin configuration. The proportion of oocytes beginning to mature (metaphase I and beyond) was found to vary significantly ($p < 0.05$) between the classes of ova. The predominant ovum type seen in culture was termed "probable nonovulatory." Table 9 examines *in vitro* maturation and fertility of these oocytes. Again, there were significant differences between classes of ova for both *in vitro* maturation and fertilization. In all categories the probable preovulatory oocytes rate the highest. The title "preovulatory" assigned by Soupart and Morgenstern may be a misnomer as these ova were recovered from all four follicle sizes described earlier.

The effects of changes in the components and environment of the culture are best examined by considering *in vitro* maturation and fertilization. Table 10 shows the results of the oxygen tension trials. *In vitro* maturation was highest for cultures maintained in the 20 and 30% oxygen atmosphere. Although numbers were low, fertilization was found to occur in all but one atmosphere. Studies of oxygen tension were conducted using medium TJK-2. In different trials, the five media were compared (Table 11) in the 20% oxygen atmosphere (5% carbon dioxide

Table 6. Follicular volume estimates from aspirated fluid and follicle surface diameters

Follicular diameter (mm)	Calculated fluid volume[a]	Fluid aspirated µl/follicle (N)[b]	Diameter calculated (mm)[c]
Preovulatory (3-4 mm with dome)	33.51	26.0 (11)	3.7
3	14.14	11.7 (59)	2.8
2	4.19	5.0 (46)	2.1
1	0.52	1.8 (31)	1.5

[a]In µl/follicle: assuming sphere formula (Volume = 4/3 π radius^3) using aspirated follicle diameter (4 mm diameter used for preovulatory calculation).

[b](N) = number of aspirates.

[c]Assuming sphere formula, given aspirated fluid volume.

Table 7. Follicular aspirate components from follicles of various diameters

Follicular diameter (mm)	Cell indices[a]		Aspirates containing gel	
	Follicle cells	Red blood cells	No./total	(%)
Preovulatory[b]	3.0[c]	6.2	5/11	(45)
3	3.5	5.9	24/59	(41)
2	3.3	5.5	7/46	(15)
1	2.4	4.9	1/31	(3)

[a]Logarithm to base 10 of number of cells (i.e., 10^3 cells, index = 3.0).

[b]Three to 4 mm with dome (preovulatory formation).

[c]Mean per follicle aspirated.

Table 8. Classification of oocyte quality based on initial observation and potential for maturation

Type of oocyte	Cumulus mass	Corona radiata cells	Vitellus	Oocytes	% of total	Maturing[a] No.	Oocytes* (%)
Degenerate or atretic	None	None or few	Darkening, vesicularization, nonhomogenous cytol and/or shrinkage or fragmentation	80	19.3	3	3.8
Probable nonovulatory	None	Compact layers	When observable sometimes germinal vesicle present	195	47.0	111	56.9
Probable preovulatory	Extensive, sticky, rounded cells and loosely applied	Loosely applied	When observable, germinal vesicle occasionally seen	140	33.7	90	64.3
			Total	415	100.0	204	49.2

[a]Includes both maturing and matured oocytes.

*Significant difference ($p < 0.05$) when analyzed by χ^2 method for type of oocyte.

Table 9. Classification of oocytes and potential for fertilization

Type of oocyte	Matured No.[a]	Matured *in vitro**	Percentage	Fertile/ matured*	Percentage
Degenerative or atretic	3	0	0	0/3	0
Probable nonovulatory	80	43	53.8	20/66	30.3
Probable preovulatory	75	69	92.0	35/68	51.5
Total	158	112	70.9	55/137	40.1

[a]Twenty-one matured ova not inseminated.

*Significant difference ($p < 0.05$) when analyzed by χ^2 method for type of oocyte.

Table 10. Effect of oxygen tension on ovum maturation and fertilization

Percentage O_2 in atmosphere	Ovum maturation		Fertilization	
	Mature/ nonatretic	Percentage	Fertilized/ mature	Percentage
0	4/18	22	1/4	25
10	4/12	33	1/4	25
20	41/87	47	18/41	44
30	6/13	46	0/6	0
95	1/9	11	1/1	100

Table 11. Comparison of medium environment of *in vitro* fertilization cultures

Medium	Total cultures	Total oocytes	*In vitro* matured		Fertilized	
			Number/ test[a]	Percentage	Fertilized/ matured[b]	Percentage
TJK-1	46	76	16/51	(31.4)	2/11	(18)
TJK-2	63	135	37/93	(39.8)	19/38	(50)
TJK-3	41	58	26/46	(56.5)	17/23	(74)
TJK-4	28	44	11/26	(42.3)	6/13	(46)
TJK-5	22	26	7/17	(41.2)	3/6	(50)

[a]Nonmature, nonatretic oocytes at culture start.

[b]Matured (metaphase II) ova exposed to motile sperm.

in air). Medium TJK-3 gave the greatest *in vitro* maturation and fertilization. Osmolarity of this medium at 38°C after equilibration with the 20% oxygen atmosphere was 311 m Osm/kg. The pH of all five media remained between 7.3 and 7.5 throughout the culture period. Medium TJK-3 was used in the study of insulin in the medium. The results are shown in Table 12. While insulin did not increase *in vitro* maturation or fertilization, it did significantly depress the formation of fibroblast monolayers in culture chambers with more than 1,000 follicle cells.

In the course of these experiments, the serial observations of individual fertilized ova were used to construct a time course of early embryonic development *in vitro* (Table 13). Observations of individual zygotes immediately before and after a change in developmental status were pooled by two-hour intervals following insemination. The median intervals for each stage through the eight-celled zygote are shown. Only 23% of the 2-polar body, 2-pronucleate zygotes cleaved during the *in vitro* culture. Lacmoid staining of noncleaving fertilized zygotes showed the expected chromatin configurations. Figure 1 shows such a 2-polar body, 2-pronucleate zygote. At the end of the culture period, this zygote was stained (Figure 2) and both polar bodies and pronuclei were found. Thirteen zygotes were transferred to recipient females prior to the end of culture. Table 14 provides a summary of these procedures. Recipient 1321 received a 2-polar body, 2-pronucleate zygote 34.1 hours after an ovulatory injection of HCG. On day 20 (18.5 days after the transfer) the corpus luteum was still present

Table 12. Effects of insulin in the *in vitro* fertilization system

	Ovum maturation		Fertility		Fibroblasting cells	
	No./non-atretic[a]	Percentage	No./mature[b]	Percentage	Chambers forming monolayer/total*	Percentage
Control	26/46	55	17/23	74	32/39	82
Insulin (10 miu/ml)	6/20	30	4/8	50	8/15	53
Insulin (100 miu/ml)	4/9	44	1/4	25	2/13	15

[a]Included only nonatretic ova (ova matured at initial time were not included).

[b]Included only ova that matured while sperm were motile in culture.

*Significant difference ($p < 0.05$) when analyzed by χ^2 method for effects of insulin.

Table 13. Time course of development of *in vitro* fertilized zygotes

Stage	No. of zygotes	Developmental stage of 50% of zygotes (hours after insemination)	
		Earliest	Observed
2-polar body-2-pronucleate	78	6	22
2-celled	18	20	40
4-celled	8	46	52
8-celled	6	52	72

[a]The earliest possible time after insemination that 50% of the zygotes could be at the given stage.

[b]The observed time, after insemination, that 50% of the zygotes had reached the given stage.

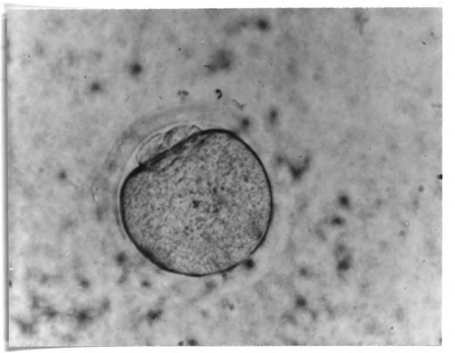

Figure 1. *In vitro* fertilized ova with 2-polar bodies (X 200).

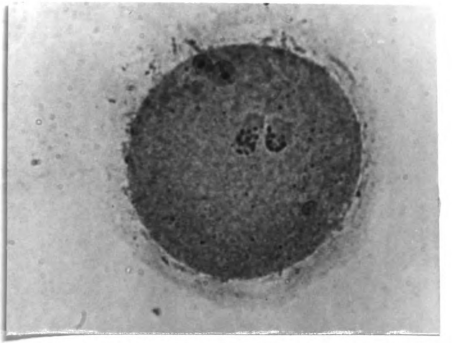

Figure 2. Ova fertilized *in vitro* stained with lacmoid to show chromatin of 2-polar bodies and 2 pronuclei (X 300).

Table 14. Results of embryo transfer in *Saimiri sciureus*

Genetic Parents		Recipient	Time of	Stage		
Female	Male	female	transfer[a]	transferred	Ovary	Treatment
1393	DUKE	1308	9.8	2PB-2PN	PRE	CS
1378	MICK	1404	10.6	2PB-2PN	PRE	HCG
1378	MICK	1399	11.5	2PB-2PN	PRE	HCG
1321	MICK	1366	22.5	2PB-2PN	CH	None
1384	DUKE	1321	34.1	2PB-2PN	CL	None
1367	MICK	1367	35.6	2PB-2PN	CL	None
1374	MICK	1374	35.9	2PB-2PN	CL	None
1391	MICK	1362	38.5	2PB-2PN	CL	None
1374	MICK	1360	36.1	2-cell	CL	HCG
1374	MICK	1312	36.4	2-cell	None	PROG
1361	DUKE	1312	37.7	2-cell	CL	None
1361	DUKE	1323	56.2	2-cell	CL	None
1303	DUKE	1302	71.6	4-cell	CL	None

[a]Hours after ovulatory HCG administration to recipient.

Key: PB = Polar body
PN = Pronucleus
PRE = Preovulatory follicle
CH = Corpus hemorrhagicum
CL = Corpus luteum
CS = Cervical stimulation
HCG = 50 i.u. injection on day four
PROG = 5 mg progesterone daily.

and the uterus was ballooned but measured only 6 mm in width. A mouse uterine-weight assay, performed on the plasma, was negative for gonadotropin. Five days later a confirmatory laparoscopy was conducted and the corpus luteum was not observed. While at least two other recipients presented signs of pregnancy, none were confirmed.

Discussion

With the use of *in vitro* fertilization system described above, fertilized squirrel monkey ova have been obtained. This system can provide a supply of early preimplantation embryos to permit the examination of the genetic safety of embryo transfer procedures in a primate model. These embryos could also be used in teratological testing to examine the hypothesis that preimplantation embryos are relatively resistant to teratogenic agents (Austin, 1973). However, ova fertilized *in vitro* are the result of a multi-step process that starts with an ovarian follicle and ends with the fertilized ovum in culture. Attrition during the procedure is high. The present results should provide for improvement of this condition in the future.

By the extensive use of laparoscopic procedures, one can maximize the usage of valuable animals, a point especially pertinent with the current shortage of nonhuman primates for research. Laparoscopic recovery of oocytes does not alter the normal development of the ovum. This finding confirmed the related human work of Edwards et al. (1969), and Morgenstern and Soupart (1972). Such procedures avoid extensive adhesions while providing for greater comfort to the animals.

An added advantage is that the aspiration procedures can be repeated many more times with laparoscopy, at least providing for an increase in the number of follicles aspirated and confirming similar observation in the Cynomologus Macaque (Dukelow and Ariga, 1976).

The size of the follicles (surface diameter) aspirated does affect the recovery. The larger follicles have more follicular fluid and more loosely confined oocytes, enhancing the recovery rate. Preovulatory follicles offer an exception to this rule. A higher proportion of aspirations from preovulatory follicles contain gelatinous or viscous fluid which blocks the needle making recovery of the oocyte more difficult. The maturity and fertility of these ova is high as would be expected if they were to be ovulated. The data presented for collection of red blood cells in the aspirated fluid indicates an increased vascularity and fragility of capillaries in the vicinity of the follicle as it increases in size. These cells do not affect the fertility *in vitro* (Kuehl, 1974) but do make observation of oocytes more difficult during the culture period. Follicle cells are diluted by a factor of ten when placed in the culture chamber. Smaller chamber volumes would decrease this dilution and perhaps improve maturation and fertility as Brinster (1970) has reported for mouse ova. Soupart (1975) has suggested that dilution of follicle cells is a cause of decreased ovum maturation *in vitro*. These results indicate *in vitro* maturation of squirrel monkey oocytes is at the same level as that achieved by Soupart's use of a two-stage maturation medium, so extra steroid supplementation of the medium may not be necessary to achieve maturation.

Soupart and Morgenstern (1973) proposed a classification system for human oocytes (shown in Table 8). They estimated potentials of nil, good, or excellent to describe oocytes that initially appear degenerate, nonovulatory or preovulatory, respectively. The results for the squirrel monkey confirm their estimates and further suggest similar potentials for the *in vitro* fertility of aspirated ova. The majority of the ova that mature in the present system do so *in vitro*. This further emphasizes the importance of the culture environment for maturation of oocytes. Two requirements for mammalian fertilization (Soupart and Strong, 1975) are the availability of mature oocytes (those having achieved the metaphase II stage of meiotic division) and the availability of capacitated sperm. The present system supports oocyte maturation as demonstrated by the finding of lacmoid-stained chromatin in the first polar body and a metaphase spread in the ooplasm. Spermatozoa have also been observed in the perivitelline space. Thus, the system supports *in vitro* capacitation. Soupart and Strong (1974) define three criteria as convincing evidence of fertilization short of a successful transfer of an *in vitro* fertilized embryo and birth of an offspring. These are (1) the presence of two or more polar bodies in the perivitelline space, (2) the presence of two pronuclei within the ooplasm, and (3) the presence of remnants of the fertilizing sperm midpiece and tail within the ooplasm. The first two of these criteria have occurred in this system. Furthermore, the development of the fertilized squirrel monkey ovum follows a similar time course through the first, second, and third cleavage divisions to the human *in vitro*

fertilized embryo in culture (Edwards, 1973). However, we were unable to find conclusive evidence of the fertilizing sperm tail and midpiece in these fertilized ova. Soupart and Strong (1974) have reported a progressive dismantling of the midpiece and tail of the fertilizing sperm in the late pronucleate stages of human *in vitro* fertilized ova, so that remnants were visible only by means of electron microscopy. As the ova in this system were cultured for 48 hours or more before fixation and staining, a similar phenomenon may account for the inability to observe the remnants of the fertilizing sperm.

The culture environment may affect both *in vitro* maturation and fertility in this culture system. The results of study of the effect of oxygen tension on maturation and fertilization are in contrast to the rabbit work of Brackett (1966) who found optimal results in culture media having a low oxygen tension but the difference is perhaps explained by the very moist atmosphere of our culture incubator. Brackett and Williams (1965) did report a high (73%) rate of fertilization when the relative humidity was 96 to 97%. Very high levels of oxygen (95%) restricted maturation although the one ovum maturing was ultimately fertilized. Edwards (1975) recommends a low level of oxygen (5%) for human *in vitro* fertilization as does Soupart (1975). This lower level is not required in the squirrel monkey system in the 0.5 ml chamber. The medium which gave the best results contained serum with added pyruvate (TJK-3) using a medium 199 base. Ham's F-10 medium contains pyruvate in the formulation, but in these trials gave the poorest results. When insulin was added to the medium, no enhancement

of fertilization was observed. Soupart's (1975) contention that additions of insulin (10 m.i.u./ml) to the medium favor follicle cell maintenance is contraindicated by the decrease in monolayer formation shown in Table 12.

The results from the transfer experiments have not, as yet, been positive. Squirrel monkeys have supported normal pregnancies following induction of ovulation (Jarosz and Dukelow, 1976). The technique of placing the zygote in the oviduct has proven to be successful. Three females were given a low dose of HCG to stimulate luteal tissue (Surve et al., 1973). Another received cervical stimulation and one was given progesterone. None of these treatments resulted in a pregnancy.

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A LAPAROSCOPIC TECHNIQUE FOR TRANSFER OF EMBRYOS IN NONHUMAN PRIMATES

Abstract

A laparoscopic technique has been developed for placement of zygotes (early preimplantation embryos) into the fimbriated end of the squirrel monkey oviduct. This technique allows for transfer of 1 to 3 µl of fluid to the oviduct *in situ* without the trauma of laparotomy.

Introduction

The first successful transfer of a fertilized ovum was by Heape (1890). He used a surgical technique to transfer two four-celled zygotes from an Angora rabbit to the oviduct of a mated Belgian rabbit. Both zygotes implanted and the young were born alive. Since that time, successful transfers have been reported for many laboratory and domestic species and several reviews have been published (Adams and Abbott, 1971; Chang and Pickford, 1969; Dzuik, 1969). Generally ova to the two-celled stage are transferred to the ampulla of the oviduct, whereas zygotes in later stages of development are transferred to the uterus of the recipient. Synchrony of the recipient to the ovum donor is necessary to obtain a viable pregnancy (Edwards and Steptoe, 1975).

Most techniques used for ovum and zygote transfer require surgical procedures to expose the oviduct. Techniques for transfer

to the uterus are either surgical (requiring laparotomy to expose the uterus) or nonsurgical (entering to the uterus via the vagina). All species in which successful transfers have been reported tend to have a flacid type uterus as compared to the muscular primate uterus. No primate species (human or nonhuman) has yet borne the product of a transfer. The techniques used in the primate have so far involved transfer to the uterus by puncture through the uterine wall or the cervix (Edwards and Steptoe, 1975). Transfers to the oviduct have not been attempted.

The goal of the present study was to develop a method for transfer of small volumes of fluid containing fertilized ova to the oviduct of a recipient host using a laparoscopic procedure to avoid the trauma of major abdominal surgery.

Materials and Methods

Animals

Squirrel monkeys *(Saimiri sciureus)* of Colombian and Bolivian origin were maintained as previously described (Kuehl and Dukelow, 1975). Mature females were assigned to ovum donor-recipient host pairs, induced to ovulate using a regime previously reported (Dukelow, 1970) with modifications during the summer anovulatory season (Kuehl and Dukelow, 1975), and synchronized with the ovulating dose of HCG (500 iu, A.P.L.; Ayerst Laboratories, New York, N.Y.). Techniques for ova recovery, *in vitro* fertilization and zygote culture have been described elsewhere (Kuehl and Dukelow, 1975; Kuehl et al., 1976).

Transfer Procedure

Recipient females were anesthesized with sodium pentobarbital (16.2 mg intraperitoneally) and laparoscoped using a technique we have previously described (Dukelow et al., 1971; Harrison and Dukelow, 1974). A second accessory probe was inserted ventrally on the side opposite the manipulatory probe (Figure 3). The ovaries were examined for ovulation points and transfers were made to the oviduct ipsilateral to the ovary with a corpus luteum. The accessory probe was withdrawn from the contralateral side and replaced with a Hartman alligator forceps (Roboz Surgical Instrument Company, Inc., Washington, D.C.). These forceps were used to manipulate the fimbria so that the opening to the ampulla was visible through the laparoscope. The zygotes were rinsed three times in fresh medium to remove motile spermatozoa. The zygotes were taken up individually in a 1 µl volume with a micropipette (Micro/pettor A, (Range: 1 to 5 µl); Scientific Manufacturing Industries, Emeryville, California). The accessory probe was withdrawn from the ipsilateral side and replaced with the micropipette (Figure 3). The tip of the micropipette was inserted into the ampular opening of the fimbria at least 1 mm deep. The plunger was pressed, forcing the zygote and 1 µl volume out of the pipette into the oviduct. The pipette was withdrawn from the ampullar opening with the plunger depressed. Following completion of the procedure, the recipient was maintained in a recumbent position for five minutes. Three females were laparoscoped and received transfers of a Trypan Blue (0.05%) solution in 1, 1, 2, 3, 4, and 5 µl volumes (one transfer per oviduct) using the procedure

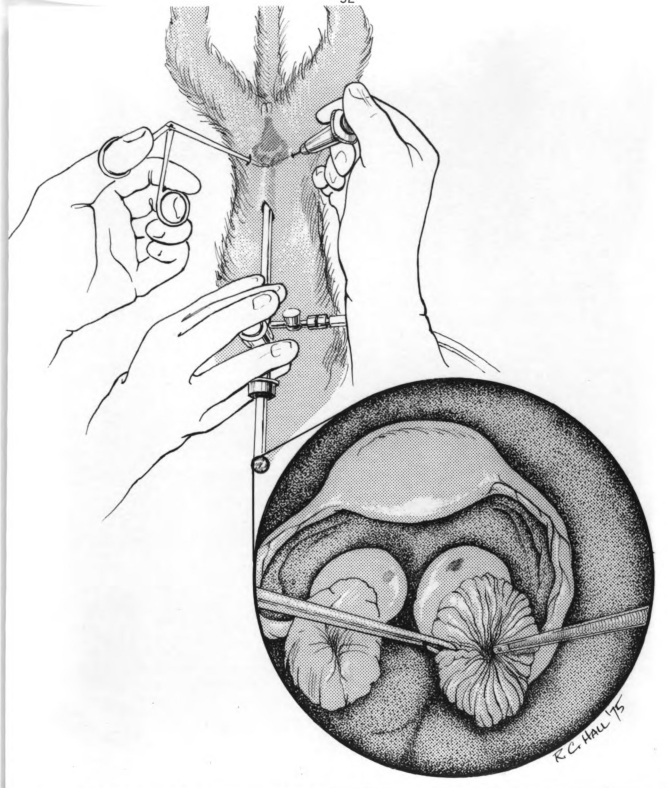

Figure 3. Diagrammatic presentation of the ovum deposition technique in the squirrel monkey.

described above. Following the dye transfers, the fimbria were examined for signs of leakage of the dye.

Results

Twelve zygotes have been transferred. Five two-celled and seven 2-polar body, 2-pronucleate *in vitro* fertilized zygotes were transferred to twelve synchronized recipients. Although some gestational changes (uterine size increases, weight gain, and increased water consumption) were noted in two animals receiving two-celled zygotes, no proven pregnancies have yet been found. In the transfers of Trypan Blue solution, 1, 2, and 3 µl of dye could be deposited at the ampullar site without leakage from the ampulla. Small amounts of dye solution were visible on the fimbria following the 4 and 5 µl volume transfers. Within fifteen seconds of the removal of the micropipette from the oviduct, all traces of dye had moved to the ampullar opening and disappeared into the oviduct. Thus the dye transfers demonstrated that small volumes used to transfer zygotes can be placed at the ampullar opening of the fimbria without loss to the peritoneal cavity.

Discussion

This report constitutes the first use of an ancillary laparoscopic procedure to deposit either fertilized ova or fluids into the oviduct *in situ*. The successful conclusion of a transfer with the birth of a live infant requires that each step of the

multiphase procedure be successful. While a variety of both laboratory and domestic species have yielded to such experimental manipulations, primates have not. This oviductal transfer technique represents one step of this multiphase procedure in the squirrel monkey to recover ova from ovarian follicles, mature and fertilize them *in vitro*, and deposit them into a foster mother.

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UTILIZATION OF COMPUTER TECHNOLOGY FOR REPRODUCTIVE STUDIES IN NONHUMAN PRIMATES

(CODATA Bulletin (accepted), 1976)

Abstract

Computer programming techniques were used to (1) aid raw data collection, handling and compilation into data banks, (2) analyze banked data for basic reproductive values and specific experimental designs, and (3) utilize reproductive values for construction and simulation of computer operated models. Raw data from *in vitro* fertilization experiments with squirrel monkeys was compiled in a master file for statistical analysis. A bank of reproduction data for Macaque monkeys was established and examined for menstrual cycle characteristics. A computer simulation model was constructed to simulate the circhoral pattern of plasma LH concentrations observed in ovariectomized rhesus monkeys. A control point of the model was found and manipulated for expansion of the model to include drug induced changes in these circhoral patterns.

Introduction

The computer has served as a useful tool in biomedical research (Lindberg, 1974). Applications in two areas, computerized record keeping and simulation modeling, are of particular interest to investigators of nonhuman primate reproduction. The value of computer simulation of

complex physiological systems has been discussed by several investigators (Schwartz, 1968; Riggs, 1970; Apter, 1974). The logistics for handling such computer simulation models have evolved along two lines. One approach has been to create new language for specific systems. Garfinkel (1968) used this approach in developing a machine-independent language for analyzing and simulating biochemical systems. This program was used to analyze experimental data from studies of rat liver cytosol gluconeogenesis (Achs et al., 1971), construct a simulation model (Anderson et al., 1971) and verify the model in later studies of rat liver dicarboxylic acid distribution (Anderson and Garfinkel, 1971). The other approach has been to use more general packaged programs. For example, the recent study by Phair et al. (1975) used the SAAM computer program (1967) to construct and modify a preliminary model of human lipoprotein metabolism. The latter approach was used to model the ovarian-pituitary interactions of the rat estrous cycle (Schwartz and Waltz, 1970) using CSMP (an IBM packaged simulation program). Plasma concentrations of estrogen and luteinizing hormone (LH) and the timing of ovulation were simulated for the rat. Other investigators have proposed and simulated similar models for the human menstrual cycle using both approaches (Vande Wiele et al., 1970; Shack et al., 1971; Cargille and Dixon, 1973). While these investigators have increased the number of variables included in their simulations (by adding other reproductive hormones, indices of follicle maturity and corpus luteum functions), none have provided for variability between experimental subjects or between cycles of a single subject. Such considerations

are needed to construct a simulation model for predicting reproductive phenomena of an individual subject. This study will present a simulation model for hypothalamic pituitary interactions in individual ovariectomized rhesus monkeys. To expand this model to include intact females, physiological data for individual animals are needed. The nonhuman primate literature does not contain much of this type of data: a problem discussed by Terry and Morrow (1972). They suggested the establishment of data banks as one possible solution. This dissertation will describe a bank of Macaque reproduction data. The establishment of this data base led the author to consider a data record system for studies of squirrel monkey reproduction.

Computerized data record systems are relatively new to biomedical research. These systems are of two general types: those for processing animal status and cost expenditures (Warren et al., 1972) and those for handling research data (Munro et al., 1972; Ransil, 1974). The major drawback to wide acceptance of these data systems has been their specificity for the originator's facilities. Recently, two systems, Primate Information Retrieval System (PIRS) (Sciabbarrasi and London, 1974) and Primate Information Management Experiment (PRIME) (PRIME Manual, 1972), have been developed to handle both research data and animal status information at primate research facilities. They are complete and flexible to cover the variety of input data, but they produce only preprogrammed status tables. While status and performance data are important to studies of reproduction, specific research projects often require one-time looks at experimental variables. Also, computer facilities large enough to handle these programs

are stocked with packaged statistical programs requiring special data structures. A program which manipulates a complex data file to produce data structures compatible with such packaged statistical programs would prove more useful. This was the direction followed in constructing the data record system described below.

Materials and Methods

All programming was written in FORTRAN (version 4.5) for use on a Control Data Corporation 6500 computer (Michigan State University HUSTLER 2 system). The programs were designed for interactive execution through a remote terminal (model 33 ASR teletype). Programs and data files were stored centrally on magnetic disk, while locally maintained card files provided security from system failure. MSU STAT system (1974) provided packaged statistical programs. Plotting was done on a Cal Comp 963 incremental pen plotter using software provided on the HUSTLER 2 system (Plotting and Graphics, 1974). The programs described below (HYPIT and DAS) are available from the Endocrine Research Unit, Michigan State University.

Computer Model

A mathematical model was formulated for hypothalamic-pituitary interactions leading to circhoral oscillation in the plasma LH concentration of an ovarectomized rhesus monkey. Literature values used in modeling and for simulation of pharmacologic blockade of these circhoral patterns were from work by Knobil and his coworkers (Atkinson et al., 1970; Dierschke et al., 1970; Bhattacharya et al., 1972). Figure 4

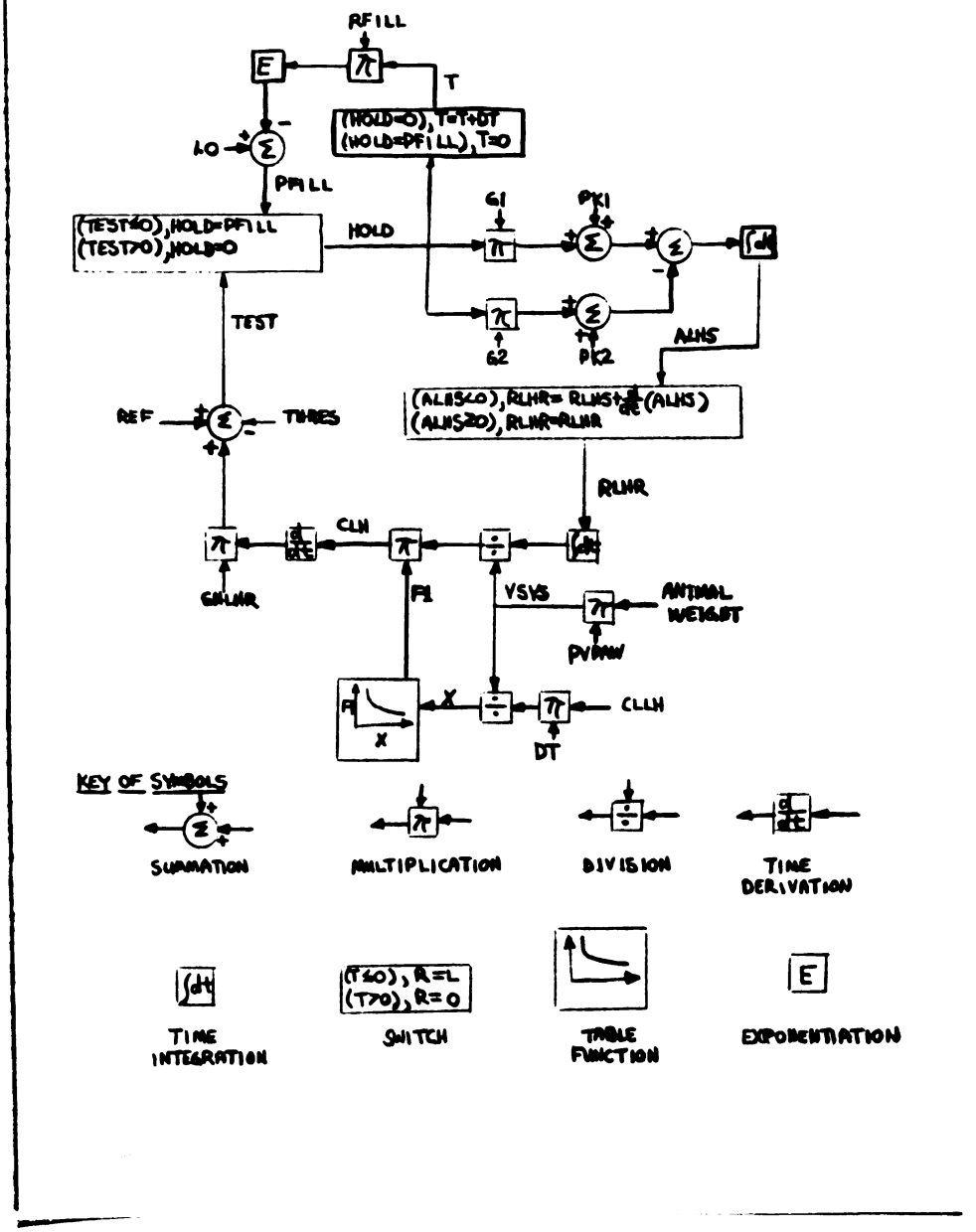


Figure 4. Flow diagram for simulation of plasma LH concentration in the ovariectomized rhesus monkey.

shows a flow chart for the computer simulation program (termed HYPIT). Variables used in the simulation are listed in Table 15 along with their initial values. Simulation was carried out in two modes. The first was to initiate variables and determine the sensitivity of the output (plasma LH concentration) to changes in control variables (THRES, RFILL, and gain factors). The second mode was to simulate an individual animal's response to a neuroleptic drug.

Macaque Reproduction Data Bank

Protocols were developed consisting of the variables shown in Table 16. These protocols were used to structure data from a *Macaca fascicularis* colony located at the Endocrine Research Unit, Michigan State University, and a *Macaca arctoides* colony of Sandoz, Ltd., Basel, Switzerland. A line of data (values for the protocol) was recorded for each menstrual cycle of every female Macaque. MSU STAT routines were used to analyze these data for cycle and menses characteristics.

Computer Record System

An 823 line FORTRAN program (termed Data Analyzer Series, DAS) was developed to manage data collected during the course of *in vitro* fertilization experiments with squirrel monkeys. DAS employs a subroutine structure which contains algorithms to (1) produce raw data forms, (2) edit the master file, (3) search the master file for user specified entries and (4) list selected data in user specified format. The master file consists of entries of data (termed cells). Three

Table 15. List of variables used in HYPIT simulation program

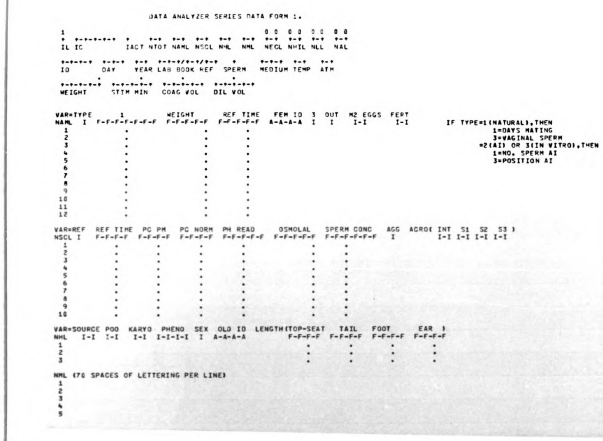
Symbol	Variable	Initial value
RFILL	Net rate of filling of GNRH stores	-1.0 %/100/hour
T	Time interval from previous GNRH release	0.0 hour
PFILL	Amount GNRH available for release (i.e., GNRH store)	0.0 %/100
HOLD	Amount GNRH in transit to pituitary receptors	0.0 %/100
G1	Gain for GNRH signal effect on synthesis of LH	300 µg/%-hour
PK1	"Basal" LH synthesis	0.0 µg/hour
G2	Gain for GNRH signal effect on release of LH	300 µg/%hour
PK2	"Basal" release of LH	0.0 µg/hour
ALHS	Amount LH available for release	0.0 µg
RLHR	Net rate of LH release	cal. µg/hour
RLHS	Net rate of LH synthesis	cal. µg/hour
ANIMAL WEIGHT	Mass of animal to be simulated	5.0 kg
PVPAW	Plasma volume per animal weight	40.0 ml/kg
VSYS	Plasma volume	200.0 ml
CLLH	Clearance of LH from plasma volume	110.0 ml/hour
DT	Increment of time, plasma is cleared before next iteration of simulation	0.02 hour
F1	Gain of CLH due to clearance of volume of plasma	calculated
CLH	Plasma concentration of LH	50.0 mg/ml
GHLHR	Gain hypothalmus LH release	-1.0 ml-hour/mg
THRES	Threshold for "firing" of GNRH neuron	12.0
REF	Reference point of threshold "firing mechanism"	0.0
TEST	A test value used to trigger "firing" of GNRH	calculated
GNRH	Gonadotropin releasing hormone	
LH	Luteinizing hormone	

Table 16. List of variables in protocols for Macaque reproduction data bank

Type of protocol (location of colony)
Female monkey number
Menses data
Menses length and type of flow
Ovulation confirmation (laparoscopy, laparotomy, or pregnancy)
Day of ovulation and side of ovulation ovary
Male monkey number (if breeding involved)
Mating date
Semen quality
Day of pregnancy test and test results
Implantation bleeding date
Length of implantation bleeding and type of flow
Mode of pregnancy termination
Date of termination
Offspring monkey number
Sex of offspring
Laboratory notebook cross-reference

types of cells are used, corresponding to the three experiment units of the *in vitro* fertilization system (the female, ova donor; the male, sperm donor; and the culture chamber containing the gametes). The structure of the cells are shown by the data forms used to record cell values (Figures 5, 6, and 7). All cells have several common features. The first line (cell descriptor line) contains information on the cell type and composition as well as a unique cell number for cross-referencing (IC). Each cell has a line for integer data and a line for decimal data unique to that type of experimental unit. These are followed by from two to five optional groups of lines providing time series or repeated observations for a treatment. All cells contain a laboratory notebook reference and entry date (year-day) which provides a reference point. A form was prepared for each experimental unit in the *in vitro* fertilization system and entered as a data cell from the remote terminal.

The DAS program prompts the user during an interactive session to respond with a command (LIST, EDIT, SEEK, HELP, STOP and FORM) which directs program execution through the proper subroutines. The LIST and EDIT commands provide management of files used by the program including updating and correcting of the master file. The SEEK allows the user to filter desired information from the master file and place it in a format for use by packaged routines or for immediate consumption as a list or table. HELP is used to remind the user of program operation during the interactive session and STOP terminates the program. The FORM statement generates new raw data forms which are printed at the



```
                    DATA ANALYZER SERIES DATA FORM 1.

1                                                    0 0  0 0  0 0   0 0
*  +-+-+-+-+-+-+  +    +-+  +-+  +-+  +-+  +-+  +-+  +-+  +-+  +-+  +-+
IL IC             IACT NTOT NAML MCCL MHL  NML  MFCL MHIL NLL  NAL

   +-+-+-+-+  +-+-+  +-+  +-+-+-+/+-+-+  +      +-+-+-+  +-+  +-+
ID            DAY    YEAR LAB BOOK REF   SPERM  MEDIUM TEMP  ATM
              +      +    +    +     +             +
   +-+-+-+-+-+  +-+-+-+-+  +-+-+-+-+  +-+-+-+-+
WEIGHT          STTM MIN   COAG VOL    OIL VOL

VAR=TYPE          I          WEIGHT      REF TIME   FEM ID  3   OUT  M2 EGGS  FERT
NAML  I  F-F-F-F-F-F-F-F  F-F-F-F-F-F  F-F-F-F-F  A-A-A-A-A  I   I    I-I      I-I        IF TYPE=1(NATURAL),THEN
  1               .             .           .                                                      1=DAYS MATING
  2               .             .           .                                                      3=VAGINAL SPERM
  3               .             .           .                                                    =2(AI) OR 3(IN VITRO),THEN
  4               .             .           .                                                      1=NO. SPERM AI
  5               .             .           .                                                      3=POSITION AI
  6               .             .           .
  7               .             .           .
  8               .             .           .
  9               .             .           .
 10               .             .           .
 11               .             .           .
 12               .             .           .

VAR=REF   REF TIME   PC PM   PC NORM  PM READ      OSMOLAL    SPERM CONC    AGG  ACROS( INT  S1  S2  S3 )
NSCL I  F-F-F-F   F-F-F-F  F-F-F-F  F-F-F-F-F  F-F-F-F-F  F-F-F-F-F-F    I          I-I I-I I-I I-I
  1       .         .        .        .           .          .
  2       .         .        .        .           .          .
  3       .         .        .        .           .          .
  4       .         .        .        .           .          .
  5       .         .        .        .           .          .
  6       .         .        .        .           .          .
  7       .         .        .        .           .          .
  8       .         .        .        .           .          .
  9       .         .        .        .           .          .
 10       .         .        .        .           .          .

VAR=SOURCE POO  KARYO  PHENO  SEX  OLD ID  LENGTH(TOP-SEAT   TAIL   FOOT     EAR  )
NML   I-I  I-I   I-I  I-I-I-I  I  A-A-A-A      F-F-F-F  F-F-F-F  F-F-F-F  F-F-F-F
  1                                              .        .        .        .
  2                                              .        .        .        .
  3                                              .        .        .        .

NML (76 SPACES OF LETTERING PER LINE)
  1
  2
  3
  4
  5
```

Figure 5. Form 1 for male squirrel monkey data.

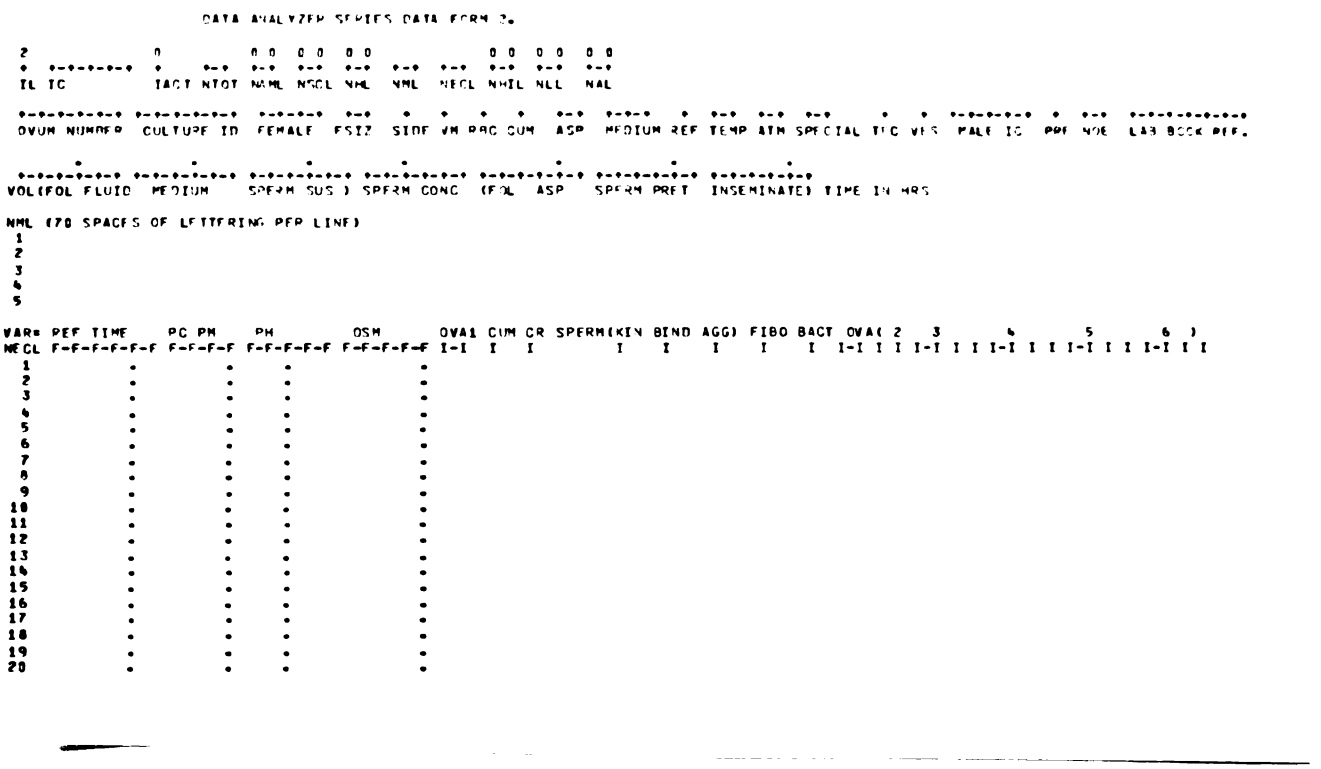


Figure 6. Form 2 for culture chamber data.

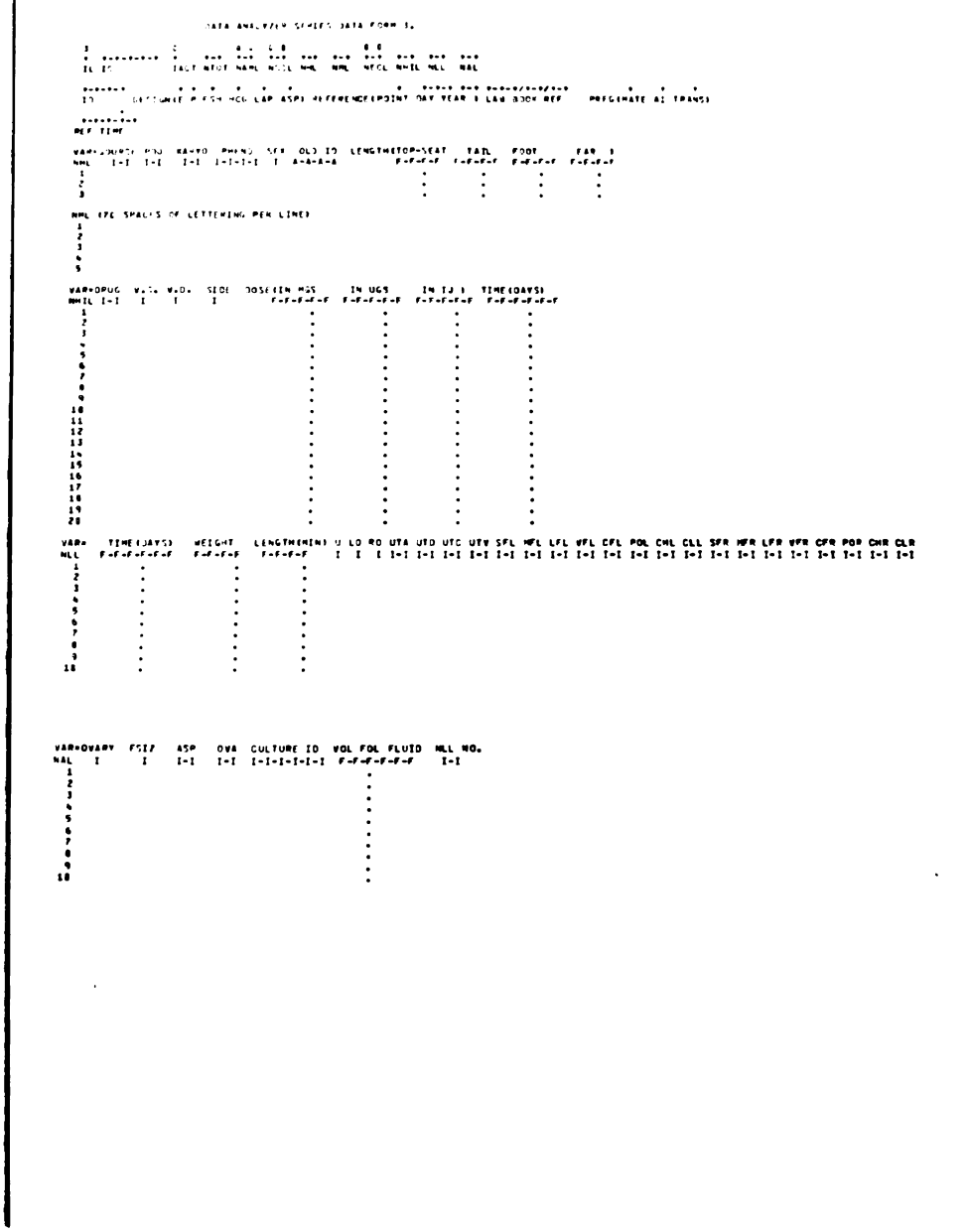


Figure 7. Form 3 for female squirrel monkey data.

central site by a high-speed line printer. The program was tested by a sample filtering of cells to collect body weight and semen qualities for a colony male, DUKE.

Results

Computer Model

A computer model (HYPIT) was used to simulate circhoral patterns of plasma LH in ovariectomized rhesus monkeys. When simulation variables were initiated to the values shown in Table 15, the period length between peaks of plasma LH concentration was 75 minutes. The THRES variable (a threshold setting in the model) was found to be a convenient control point for the period length (Table 17). In a simulation of the action of haloperidol, a neuroleptic drug, THRES was modified to produce the simulated pattern shown in Figure 8. This pattern agrees with that experimentally derived using 0.5 to 1 mg/kg haloperidol in ovariectomized rhesus monkeys (Bhattacharya et al., 1972).

Macaque Reproduction Data Bank

The data bank was established using reproduction protocols for two species of Macaques. Each colony has contributed five years of reproduction data to the bank. A total of 2,309 reproductive cycles were included for the two species. Specific results, especially concerning menstrual cycle characteristics and ovulation time in *M. fascicularis* have been described elsewhere (Dukelow, 1975).

Table 17. Period lengths (minutes) of oscillation of simulated LH patterns using different values for THRES

THRES	Period (minutes)
3	204[a]
5	150
8	112
9	102
10	93
11	84
12	75
13	67
14	60
15	54
16	49
19	35
21	30

[a]Measured as the mean for five cycles from 10 minute sampling intervals.

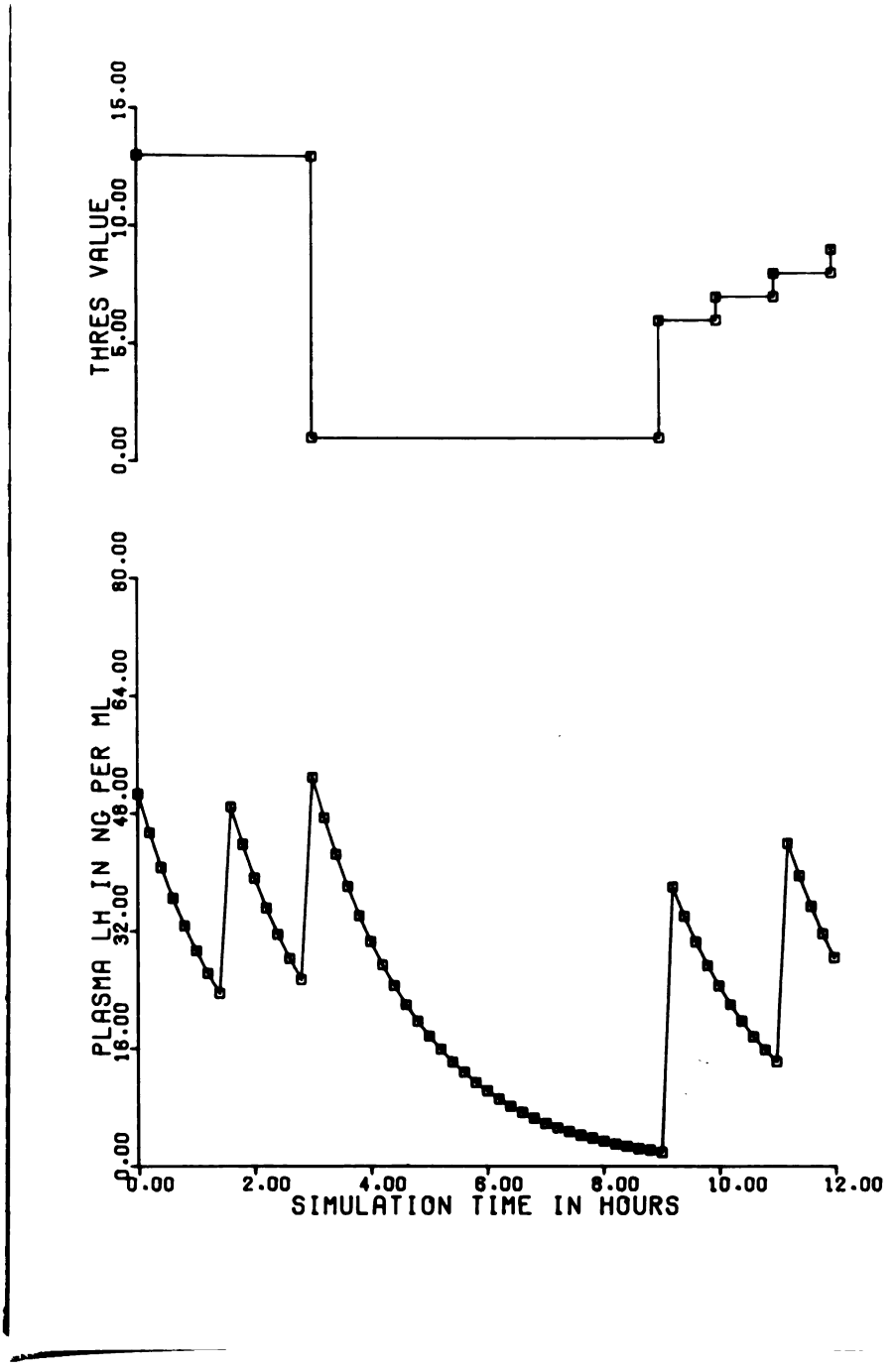


Figure 8. Results of simulation of pharmacologic blockade of circhoral oscillations of LH by alteration of THRES variable.

Computer Record System

The computerized record system (DAS) was established with 550 cells of data from *in vitro* fertilization studies. The system was utilized to examine observed seasonal changes in a male used to provide sperm. Male squirrel monkeys have been shown to undergo seasonal weight change, in wild and semi-natural conditions, which have been correlated to testis weight and changes in spermatogenesis (DuMond and Hutchinson, 1967; Baldwin, 1970; Nadler and Rosenblum, 1972). In our laboratory one particular male, DUKE, has been electro-ejaculated and weighed regularly for approximately two years. The program was used to filter from the master file a listing of the weight, volume of ejaculate, and number of sperm collected throughout DUKE's history. The listing was structured for use in a plotting routine and the results are shown in Figure 9. A listing of the data was also made. Other results using this system to compile oocyte data are found on page 24 of this dissertation.

Discussion

A simulation model has been presented for the hypothalamic-pituitary interaction responsible for the circhoral LH plasma concentration pattern in ovariectomized rhesus monkeys. A simulated drug interaction yielded results similar to those seen experimentally. The pattern of depressed circhoral surges of LH release was also observed experimentally with the infusion of estradiol into ovariectomized rhesus monkeys (Yamaji et al., 1972). Using the THRES variable in

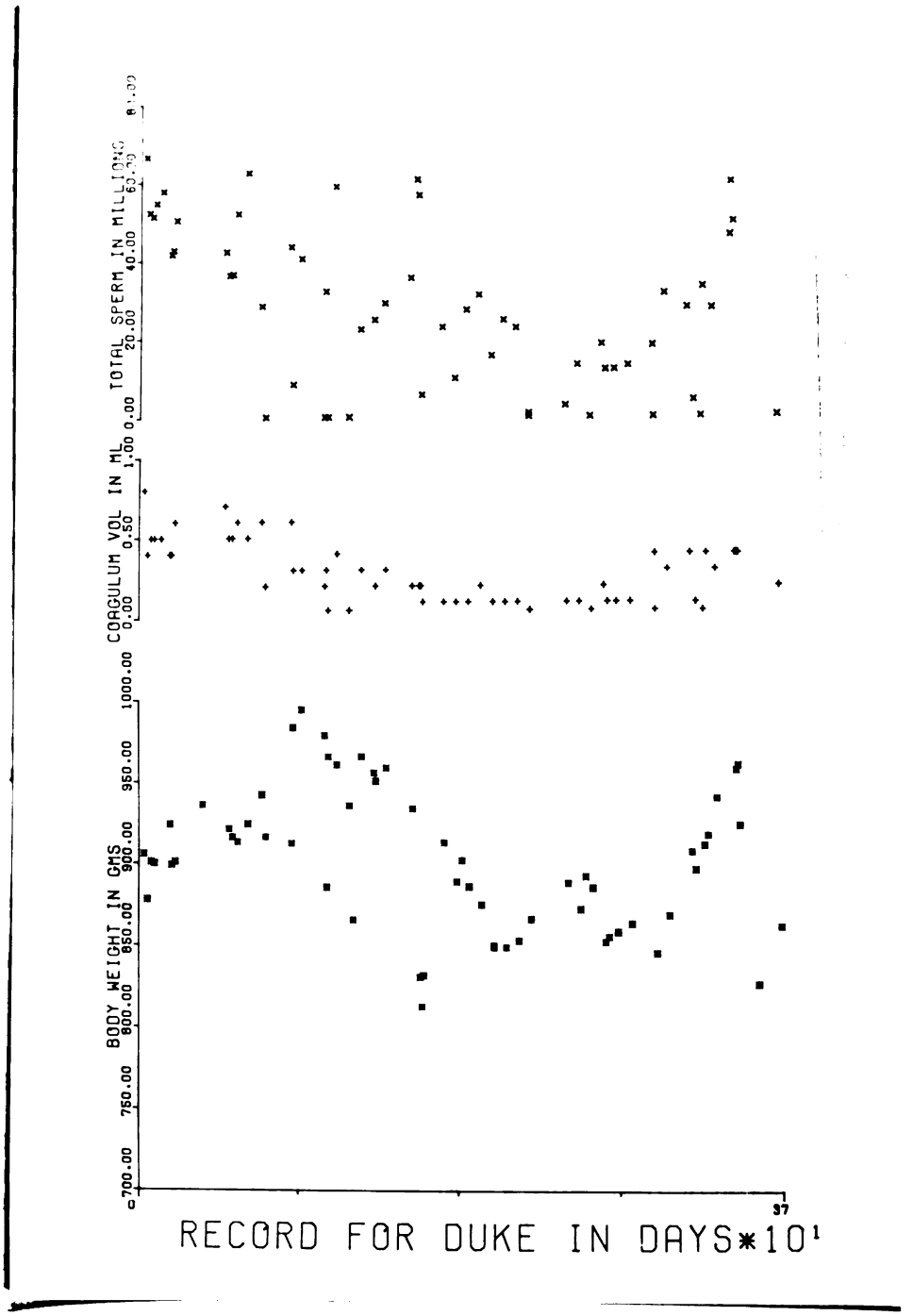


Figure 9. Seasonal changes in weight and semen characteristics for laboratory maintained male squirrel monkey.

the simulation model as a control point for this action of estrogen adds another dimension to this model.

The data bank of Macaque data has been used to exchange data between two laboratories. While the Endocrine Research Unit colony is used predominantly in laparoscopic studies to determine ovulation times, the Sandoz Ltd. colony is used in mating studies for production of timed pregnancies. These types of data compliment each other and can be combined to increase understanding of Macaque reproduction. The computerized banking of data was extended to research data. Placing complex *in vitro* fertilization data in such a system provides three advantages: (1) masses of data can be quickly available for compilation of tables and listings, (2) algorithms can be provided that interactively form data structures for statistical treatment, and (3) compilation of historical information can easily be made (i.e., information on the entire research life of a given animal can be obtained in a matter of minutes). This latter advantage is particularly important for nonhuman primate reproduction research. Due to the high cost of these animals and the nonterminal nature of the research, experimental subjects are often maintained for several years and used in many experiments. Also some phenomena, such as seasonal weight changes, may only become apparent after individual animals have been studied for several years.

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SUMMARY AND CONCLUSIONS

Saimiri sciureus was used as a model nonhuman primate for investigations of variables involved in *in vitro* fertilization and embryo transfer. Associated problems of development of a transfer technique and data handling were also studied. The following conclusions are indicated by the data obtained:

1. A laparoscopic technique was developed which allows placement of from 1 to 3 µl of fluid into the ampulla of the squirrel monkey oviduct.
2. A computer simulation model can be constructed and used to simulate circhoral patterns of plasma LH in ovariectomized rhesus monkeys.
3. A computerized data bank can be established for exchange of Macaque reproduction data.
4. A computer program can be used to compile and examine *in vitro* fertilization data for specific experimental designs, or historical data.
5. Fertility of male squirrel monkeys varies between individuals.
6. There are differences between follicles in the proportions of oocytes recovered and in proportions of recovered oocytes that are atretic.
7. Oocytes can be classified by cellular investments with regard to potential to mature and be fertilized *in vitro*.

8. Insulin added at the 10 m.i.u. or 100 m.i.u. per ml level does not alter maturation or fertilization *in vitro*.
9. The time course for *in vitro* development of fertilized zygotes is similar to that of rhesus monkeys and humans.
10. Transfer of ova from the squirrel monkey, fertilized *in vitro*, has not resulted in a pregnancy.

APPENDIX A

PREGNANCY DIAGNOSIS OF *SAIMIRI SCIUREUS*

APPENDIX A

PREGNANCY DIAGNOSIS OF *SAIMIRI SCIUREUS*

A reliable determination of pregnancy is necessary in experiments involving transfer of fertilized ova, so that recipients aborting or resorbing embryos are not misclassified as nonpregnant. Table 18 summarizes previously reported characteristics of gestation (squirrel monkey) which have been used to diagnose pregnancy. The first two, uterine size increase and chorionic gonadotropin concentration in the plasma or urine, were found to occur early in the first trimester at the time the embryo is in the primitive streak stage (embryonic stage VIII) (Goss et al., 1968). In the course of their embryological studies, Goss et al. (1968) reported the most objective indication of pregnancy in the squirrel monkey to be an enlarged (approximately 20 mm) and slightly hyperemic uterus with an ovary having a corpus luteum. These observations were made using laparotomy 20 to 25 days after conception. Nathan et al. (1966) used a rat-uterine-weight assay for chorionic gonadotropin to diagnose pregnancy from 20 to 105 days after conception. There were two false positive reactions of 23 tests and no false negative reactions. Castellanos and McCombs (1968) were successful in early diagnosis of pregnancy using a mouse-uterine-weight assay to detect elevated urinary gonadotropins. They diagnosed 11 of 17 females pregnant. Only two

Table 18. Characteristics of gestation in *Saimiri sciureus*

Characteristic	Days after conception	References
Uterine size increase (by laparotomy)	20 to 25	Goss et al., 1968
Chorionic gonadotropin:		
(a) plasma	20 to 105	Nathan et al., 1966
(b) urine	28	Castellanos and McCombs, 1968
Uterine size increase (by palpation)	42 to 56	Rosenblum, 1968
	49	Hopf, 1967.
	61	Nathan et al., 1966
Diabetes insipidus	60	Clewe, 1969
	60 to 135	Travis and Holmes, 1974
Maternal weight gain	60 to 95	Hopf, 1967
	67 to 147	Travis and Holmes, 1974
Visual abdominal enlargement	74 to 102	Hopf, 1967
Fetal skeleton by x-ray	81	Nathan et al., 1966
	84	McKim et al., 1972
	102	Hopf, 1967

pregnancies went to term; the remaining nine females resorbed their embryos before the ninth week of pregnancy.

Female squirrel monkeys in each of five groups (Table 19) were tested for pregnancy using at least one of four methods. The first was examination of the reproductive tract with the laparoscope. Pregnancy was diagnosed as positive when the uterus was found to be enlarged, hyperemic, and with a corpus luteum on an adjacent ovary. Figure 10 provides a comparison of uterine width dimensions for pregnant and nonpregnant animals. The width was taken as the distance between tubal-uterine junctions measured to the nearest millimeter. With this measure alone, pregnancy could be accurately diagnosed 100% of the time if the width was greater than 10 mm. Animals with uteri 8 to 10 mm in width were subjected to a confirmatory laparoscopy. The remaining three methods involved examination of either urine, collected by suprapubic puncture, or plasma, collected by femoral venipuncture, for chorionic gonadotropin with one of the three assays shown in Table 20. A preliminary test of the three methods was performed with (1) squirrel monkey urine collected from females 8 to 12 hours following a 500 iu intramuscular injection of HCG (A.P.L., Ayerst Laboratories, New York, N.Y.), (2) urine collected 8 and 10 days after the HCG injections, and (3) 0.15 molar sodium chloride solution with 1 iu of HCG per ml. The results in Table 20 show that the commercial human pregnancy test did not detect the HCG in any of three early urine samples that gave positive reactions by the other two methods. Table 21 provides a comparison based on the number of incorrect pregnancy

Table 19. Groups of female squirrel monkeys undergoing pregnancy diagnosis

Category	Time of test[a]
Nonpregnant	1 to 56 days post-HCG injection
Nonpregnant	at random intervals for non-HCG injected females
Possibly pregnant	at 30 day intervals during natural mating
Possibly pregnant	20 to 29 days post-HCG injection combined with either: artificial insemination, natural mating, or embryo transfer
Possibly pregnant	21 days after the end of natural mating

[a]HCG injection: 500 iu of Human Chorionic Gonadotropin (A.P.L., Ayerst Laboratories, New York, N.Y.) injected as an ovulating dose as part of ovulation induction regime used in this species (Dukelow, 1970; Kuehl and Dukelow, 1975).

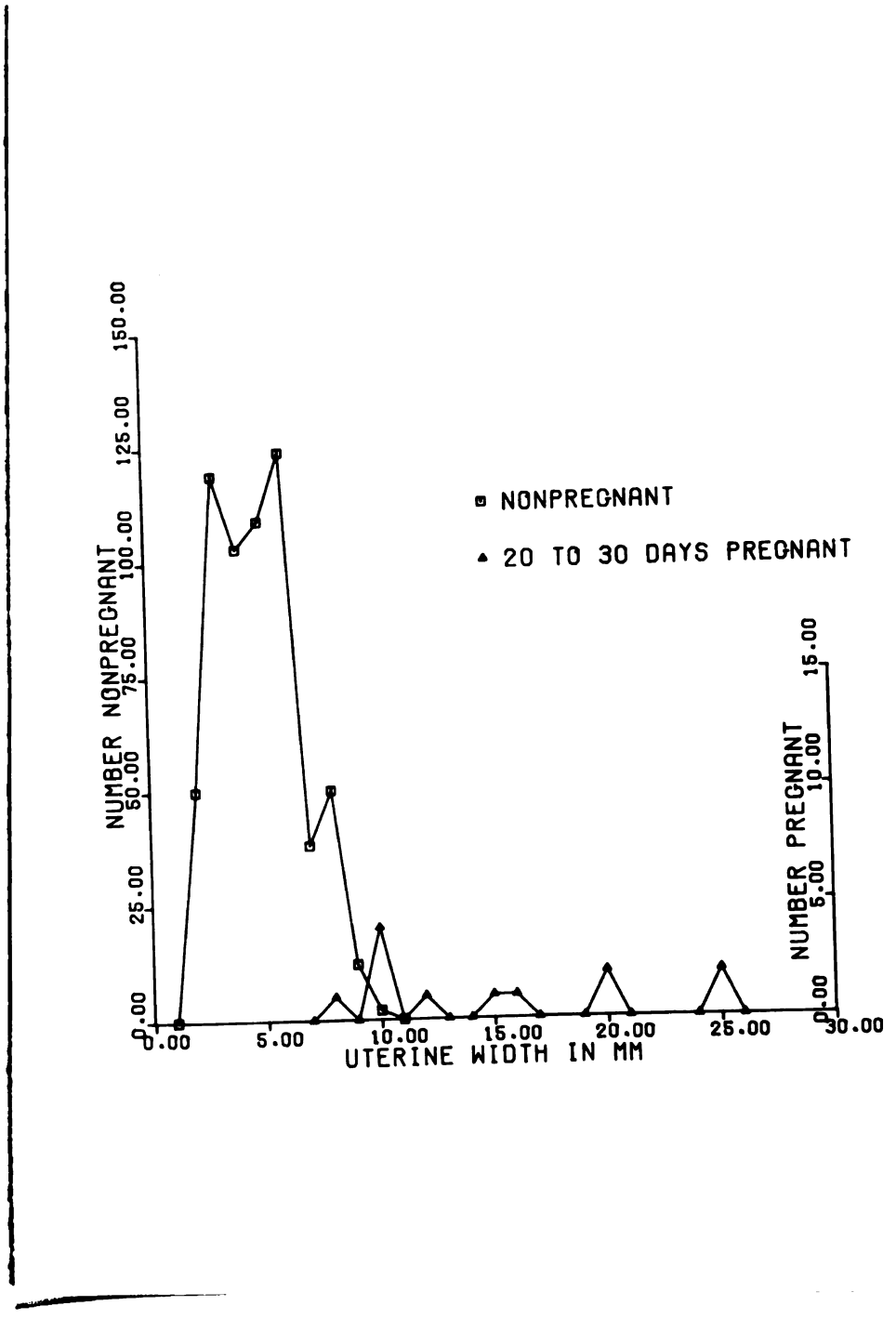

Figure 10. Frequency distribution of uterine width dimension used for pregnancy diagnosis.

Table 20. Chorionic gonadotropin assays

Method	Detection of HCG in Urine[a] 8-12 hrs	Urine[a] 8 and 10 day	Saline 1 iu/ml
Bioassay			
Mouse Uterine Weight[b]	Positive	Negative	Positive
Immunoassay			
Commercial Human Test[c]	Negative	Negative	Positive
Sub-Human Primate Test[d]	Positive	Negative	Positive

[a]Urine of squirrel monkey injected with 500 iu HCG collected 8 to 12 hours or 8 and 10 days later.

[b]With 20 day Swiss-Webster mice using method of Wilson et al., 1970.

[c]Prognosticon Immuno-Diagnostic Pregnancy Qualitative Test, Organon Inc., W. Orange, N.J.

Table 21. Comparison of diagnostic methods

Method	Total	Number of tests: False Positive[a]	False Negative
Laparoscopy	220	0	0
Mouse Uterine Weight	18	1	0
Commercial Human Pregnancy Test	8	3	0
Sub-Human Primate Test	13	2	0

[a]All false positive reactions were from 20 and 22 day plasma.

diagnoses for each of the four methods. The false positive reactions found with plasma samples might be due to a cross reaction of squirrel monkey pituitary gonadotropin. No false positive reactions resulted from tests of squirrel monkey urine. Laparoscopic examination of the reproductive tract clearly is the method of choice. Ten pregnancies were detected within a month of conception with this method.

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APPENDIX B

COMPUTER PROGRAM FOR MAINTENANCE OF INDIVIDUAL ANIMAL RECORDS IN A NONHUMAN PRIMATE COLONY

APPENDIX B

COMPUTER PROGRAM FOR MAINTENANCE OF INDIVIDUAL ANIMAL RECORDS IN A NONHUMAN PRIMATE COLONY

The nonhuman primate colony at the Endocrine Research Unit consists of animals used in a variety of nonterminal studies. Their responses to a variety of treatments as well as basic health statistics (especially body weights) are recorded as citable entries in laboratory notebooks by the investigators using them. A computer program was developed to maintain a file of animal records for this nonhuman primate colony. The program and associated data file comprise the Colony Assignment Record system (CAR). Like the Primate Information Retrieval System (PIRS) (Sciabbarrasi and London, 1974) and the Primate Information Management Experiment (PRIME) (PRIME Manual, 1972), the CAR system utilizes the concepts of a permanent master file, to contain all animal records, and unique animal identification numbers, to allow access to individual animal records. The CAR system was designed to be used by the investigators in conjunction with a laboratory notebook numbering system used for the past six years. The objectives of the CAR system were (1) to maintain a listing, by animal, of all laboratory notebook entries at a common site, readily accessible to all investigators and (2) to store both current and historical information on each animal.

The program consists of 165 lines of FORTRAN, version 4.5, and contains no subroutines. The master file of animal histories is stored as an alphanumeric permanent file. Both the program and master file are stored on magnetic disks at the central site, a CDC 6500 computer located at the Computer Center of Michigan State University. The CAR program is operated by HUSTLER 2, MSU's operating system, in conjunction with MISTIC 2 (The Michigan State Interactive Computing System). The investigator communicates with MISTIC 2, by telephone lines, from the remote site, a model 33 ASR teletype located at the Endocrine Research Unit. Both the program and master file are attached for use by the investigator at the beginning of each interactive session. MISTIC 2 then loads the program and begins execution. The program was designed for interactive use and responds to the six CAR commands shown in Table 22. A sample interaction with the program is shown in Figure 11. The investigator responses are underlined. Using the FILE command, new data can be added to the master file from a previously supplied tape. In this case, no data was to be filed. The LIST command can be used to provide the investigator with a complete listing of the master file and the current project assignment of every squirrel monkey in the colony. Each project is assigned a number which is termed a pool. There are currently nine pools, but more can be added to a maximum of 99. The SEEK command is used to provide a remote site printout of the history of any animal on file. The illustration is for BONN, an infant female. Each of the seven lines is an entry containing the animal's name, sex code, species code, pool code, location code, date

Table 22. CAR system commands

Command	Special user action[a]	Program action
FILE	Supply new status data	Adds new data to master file
LIST	Dispose copy to central site, if required	Copies master file at terminal or central site
SEEK	None	Asks for animal identity and provides complete history
HELP	None	Provides brief explanation of program operation
FORM	Dispose copies to central site	Copies data forms for use in structuring data for filing
STOP	None	Terminates current interaction with program

[a]Any actions other than answering questions posed by program during interactive session.

```
    WELCOME TO CAR,COLONY ASSIGNMENT RECORDS.HAVE A
    NICE STAY. DO YOU NEED HELP?
YES
    THIS PROGRAM KEEPS TABS ON MONKEY RESEARCH ASSIGNMENT.
    ANIMALS ARE PLACED INTO POOLS AND LISTINGS OF THESE POOLS
    CAN BE PRODUCED INTERACTIVELY.THE PROGRAM RESPONDS TO THE
    FOLLOWING COMMANDS FILE,LIST,SEEK,HELP,STOP,FORM,
    FOR FURTHER HELP SEE TOM KUEHL OR THE CAR MANUEL.
    YOUR WISH IS MY COMMAND.JUST ENTER YOUR DESIRE.
FILE
    TO FILE DATA YOU MUST HAVE ENTERED A TAPE2 WITH
    CORRECTLY FORMATTED DATA IN ORDER .HAVE YOU?
NO
    YOUR WISH IS MY COMMAND.JUST ENTER YOUR DESIRE.
LIST
    SHOULD OUTPUT BE DISCONNECTED? YES

 POOL  1 =  0,POOL  2 = 41,POOL  3 =  9,POOL  4 =  2,POOL  5 =  0,
 POOL  6 = 19,POOL  7 = 17,POOL  9 =  0,POOL  9 =  4,POOL 10 =  0,


    YOUR WISH IS MY COMMAND.JUST ENTER YOUR DESIRE.
SEEK
    WOULD YOU LIKE A COMPLETE CURRENT STATUS LIST?
NO
    OH,MASTER!WHICH ANIMAL SHALL IT BE?(0="NONE") BONN
BONN  4   1    1    1  10/ 9/75   BORN TO 1406 AND JUAN
BONN  4   1    9    2  11/12/75   BROUGHT INSIDE(LOOKS BOLIVIAN)
BONN  4   1    9    2  11/12/75   WEIGHT=179 GMS
BONN  4   1    9    2  11/19/75   WEIGHIN TJK-14-19(205 GMS)
BONN  4   1    9    2  11/26/75   WEIGHIN TJK-15-33(219 GMS)
BONN  4   1    9    2  12/ 3/75   WEIGHIN TJK-15-33(235 GMS)
BONN  4   1    9    2  12/10/75   WEIGHIN TJK-15-33(241 GMS)
    OH,MASTER!WHICH ANIMAL SHALL IT BE?(0="NONE") NONE
    YOUR WISH IS MY COMMAND.JUST ENTER YOUR DESIRE.
FORM
HOW MANY FORMS? (I2)=01
    FORMS ON OUTPUT.TO GET,"DISPOSE,OUTPUT,OR."
    YOUR WISH IS MY COMMAND.JUST ENTER YOUR DESIRE.
STOP
    THAT CONCLUDES CAR. HAVE A NICE DAY.
      END  CAR
```

Figure 11. Sample interactive session with CAR program.

COLONY ASSIGNMENT RECORD FORM
ENDOCRINE RESEARCH UNIT,MSU

THE CAR PROGRAM REQUIRES THE USE OF CODES TO INPUT SOME BASIC STATUS DATA, WHILE ALLOWING A COMMENT SECTION FOR INVESTIGATOR USE. IT IS SUGGESTED THAT

A LAB BOOK REFERENCE AND BRIEF (1 OR 2 WORD) COMMENT OR DATUM BE PLACED IN THIS SECTION FOR LATER USE IN COMPILATION OF THE ANIMAL HISTORY.
ONE ANIMAL PER FORM PLEASE. INPUT OF DATA WILL BE MADE FASTER.

NAME	SEX	SPECIES	POOL	LOCATION	DATE	COMMENT(40 SPACES)
• • • •	•	•	• •	• •	• • • • • •	•
• • • •	•	•	• •	• •	• • • • • •	•
• • • •	•	•	• •	• •	• • • • • •	•
• • • •	•	•	• •	• •	• • • • • •	•
• • • •	•	•	• •	• •	• • • • • •	•
• • • •	•	•	• •	• •	• • • • • •	•
• • • •	•	•	• •	• •	• • • • • •	•
• • • •	•	•	• •	• •	• • • • • •	•
• • • •	•	•	• •	• •	• • • • • •	•
• • • •	•	•	• •	• •	• • • • • •	•

FOLLOWING IS COMPLETE LIST OF CODES
SPECIES(1=S. SCIUREUS,2=M. FASICULARIS)
SEX(1=MALE,2=FEMALE,3=INFANT-MALE,4=INFANT-FEMALE)
POOL(1=OUTSIDE ENVIRON PROJECT
2=IN VITRO FERTILIZATION PROJECT
3=LAPAROTOMY ADHESIONS
4=PREGNANT
5=NATURAL CYCLE PROJECT
6=RESTING POOL
7=UTERINE FLUSH PROJECT
8=QUARANTINE
9=LACTATION)
LOCATION(1=OUTSIDE COMPOUND,2=INSIDE STEEL CAGE,
3=INSIDE GANG CAGE)
IF NO POOL,INDICATE WITH ZERO,NEW POOLS TO BE ADDED BY TJK ON DEMAND

Figure 12. Sample CAR system form.

of the entry and investigator comment. The HELP command generates a brief description of the program and the legal commands. The FORM command generates the data forms to be used by the investigator to add entries to the master file. A sample form is shown in Figure 12. The codes for each coded category are given at the bottom of the form for easy investigator use. When enough entries are gathered by the investigator, they are typed onto a paper tape (TAPE 2) the way they appear on the data form. These entries are then filed as described above. The final command is STOP. This will terminate the interactive session with the program. The investigator can then dispose the output, a list or group of forms, to the central site for printout by the high speed line printer.

The CAR system went into use on May 20, 1975, and holds records for all the squirrel monkeys at the Endocrine Research Unit.

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APPENDIX C

PUBLICATIONS BY THE AUTHOR

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PUBLICATIONS BY THE AUTHOR

Full Papers

- A Restraint Device for Electroejaculation of Squirrel Monkey *(Saimiri sciureus)*, by Thomas J. Kuehl and W. Richard Dukelow. *Laboratory Animal Science 24:* 364-366, 1974.

- Ovulation Induction During the Anovulatory Season of *Saimiri sciureus*, by T. J. Kuehl and W. R. Dukelow. *Journal of Medical Primatology 4:* 23-31, 1975.

- Fertilization *In Vitro* of Squirrel Monkey *(Saimiri sciureus)* Follicular Oocytes, by T. J. Kuehl and W. R. Dukelow. *Journal of Medical Primatology 4:* 209-216.

- Nonhuman Primate *In Vitro* Fertilization and Embryo Transfer: Their Relationship to Birth Defect Research, by T. J. Kuehl, D. E. Wildt and W. R. Dukelow. *Advances in Medical Primatology* (in press), Plenum Press, 1976.

- *In Vitro* Fertilization of Nonhuman Primates, by W. R. Dukelow and T. J. Kuehl. *La Fécondation; Colloque de la Société Nationale pour L'Étude de la Stérilité et de la Fécondité*, pp. 67-80, 1975.

- A Laparoscopic Technique for Transfer of Embryos in Nonhuman Primates, by T. J. Kuehl and W. R. Dukelow. *Journal of Medical Primatology* (in press), 1976.

Abstracts

- See the following pages for texts of abstracts.

Fed. Proc. 32:283A, 1973

OVULATION-MENSTRUAL CYCLE RELATIONSHIPS IN *MACACA FASCICULARIS*[1]

J. M. R. Rawson, T. J. Kuehl, and W. R. Dukelow
Endocrine Research Unit, Michigan State University
East Lansing, Michigan 48823

Using laparoscopy in nonhuman primates facilitates the observation of follicular development to the moment of rupture and cumulus mass extrusion and also the study of such temporal relationships as the lengths of the follicular and luteal phases of the cycle. 115 cycles were observed in 17 regularly cycling *M. fascicularis*. The mean cycle length was 30.8 ± 1.0 day with a median and mode of 30 days. In 78 cycles where follicular development was seen, 38 (48.7%) occurred on the left ovary and 40 (51.3%) on the right. In 19 cases where the time of ovulation was defined within a 24 hr. period, the follicular phase was 14.0 ± 1.1 days and the luteal phase was 15.6 ± 1.8 days. In 5 animals where ovulation was observed during paired consecutive cycles, 63.6% had ovulations occurring on opposite ovaries compared with 36.4% on the same ovary. In 3 animals where ovulation was observed, adhesion of the cumulus mass to the ovarian surface was noted. Laparoscopy and Sernylan anesthesia did not affect normal ovulation. One animal, subjected to anesthesia and venapuncture for 36 consecutive days and

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[1]Presented at the Fed. of Amer. Soc. for Exp. Biol. meeting, Atlantic City, New Jersey, April 15-20, 1973.

several laparoscopies, ovulated, conceived on Day 16 of the cycle (Day 3 of anesthesia and v.p.), and delivered normally on Day 169 of gestation.

A RESTRAINT DEVICE FOR SEMEN COLLECTION OF SQUIRREL MONKEYS

(SAIMIRI SCUIREUS)[1]

F. E. Howe, T. J. Kuehl, and W. R. Dukelow
Center for Laboratory Animal Resources
Michigan State University
East Lansing, Michigan 48823

A restraint device designed to allow a technique to capture, restrain and collect semen from a squirrel monkey without assistance. Male squirrel monkeys are effectively restrained using this device with very little danger of biting the handler and no danger of injury to the animal during the electroejaculation procedure.

Other procedures such as oral dosing, collecting blood, tuberculin testing and taking rectal temperature can be performed.

The construction of this device is simple and inexpensive. Material such as wood and foam rubber are used. A more permanent material such as acrylic plastic could be used in place of the wood.
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[1]Presented at the 24th Annual Mtg. Amer. Assoc. for Lab. Anim. Sci., Miami Beach, Florida, October 1-5, 1973.

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OVULATION INDUCTION AND *IN VITRO* FERTILIZATION IN SQUIRREL MONKEYS[1]

T. J. Kuehl and W. R. Dukelow
Endocrine Research Unit, Michigan State University
East Lansing, Michigan 48823

Captive squirrel monkeys *(Saimiri sciureus)* show a seasonal response to a subtle ovulation induction regime of 5 days progesterone, 4 days FSH (1 mg) and a dose of HCG (500 i.u.), the minimal response occurring from July to September. A 3 x 3 factorial design with 18 adult female monkeys was used to determine the effects of increasing doses of HCG (500, 1000 or 1500 i.u.) and increasing dose and duration of FSH (1 mg for 4 days, 2 mg for 4 days, or 1 mg for 5 days) on ovulation between early July and mid-October. An increase in either dose or duration of FSH significantly increased the ovulation. Increasing HCG was ineffective. For *in vitro* fertilization adult monkeys were induced to ovulate without regard to natural cycle. Laparotomies were 4 to 12 hr after HCG and follicular oocytes were recovered in 80% TC-199 and 20% fetal calf serum. Semen ejaculates were mixed in the medium and the sperm suspension and oocytes cultured at 37°C in a 5% CO_2/air environment. Of 654 follicles aspirated, 155 oocytes were recovered. Fifty

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[1]Presented at the Fed. of Amer. Soc. for Exp. Biol. meeting, Atlantic City, New Jersey, April 7-12, 1974.

developed to the first polar body stage and 14 of the 50 were fertilized (28%). Seven of the 14 fertilized ova reached the 2-polar body, 2 pronucleate stage and the rest were 2 or more cells.

NONHUMAN PRIMATE *IN VITRO* FERTILIZATION AND EMBRYO TRANSFER: THEIR RELATIONSHIP TO BIRTH DEFECT RESEARCH

T. J. Kuehl, D. E. Wildt, and W. Richard Dukelow
Endocrine Research Unit, Michigan State University
East Lansing, Michigan 48823, USA

Because of the adaptability of *Saimiri sciureus* to laboratory procedures and the background of information available on this species relating to ovulation and semen collection, we have developed a system capable of allowing *in vitro* fertilized *S. sciureus* ova to develop to at least the four-cell stage.

We collect semen by electroejaculation and divide the seminal coagulum for dissolution in culture-slide compartments. The fertilization medium (TC-199) includes 20% agamma fetal calf serum. To date we have been able to culture sperm and ova for periods up to 48 hours with strong sperm motility either in solution or when attached to the zona pellucida. Follicular growth is stimulated by a regime of 1 mg FSH daily for 4 days followed by 500 i.u. HCG, i.m. This is an adaptation of our previously published regime for ovulation in this species during the period from October through June. During the other 3 months it is necessary to either double the amount of FSH injected or extend administration to 5 days.

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[1]Presented at the Jerusalem Conference on Primatology, Jerusalem, Israel, Feb. 24-26, 1975.

For 38 animals, 273 follicular oocytes were recovered by aspirating 654 follicles for an average recovery of 42% or 7.2 oocytes per animal. Of the aspirated oocytes, 20% were found to be fragmented. The remaining 247 oocytes were incubated in 65 cultures and 50 developed to the first polar body stage. Fourteen of the 50 matured ova (28%) were determined to be fertilized and ranged in development between two polar body, two pronucleate and four-cell embryos.

In vitro fertilization offers the research worker many unique opportunities for investigation of drug effects on the sperm, ovum and embryo during the preimplantation stages of pregnancy. Since it is at this time that cytogenetic damage frequently occurs, the technique has special teratological application. Recent discoveries relating to different karyotypes of the subspecies of *Saimiri*, coupled with successful *in vitro* fertilization and embryo transfer, provide valuable tools for studies of genetic damage and developmental effects.

SQUIRREL MONKEY FOLLICULAR OOCYTE RECOVERY, *IN VITRO* FERTILIZATION, AND EMBRYO TRANSFER VIA THE LAPAROSCOPE[1]

T. J. Kuehl and W. R. Dukelow
Endocrine Research Unit, Michigan State University
East Lansing, Michigan 48823

The squirrel monkey *(Saimiri sciureus)* was chosen for this study of *in vitro* fertilization and embryo transfer in nonhuman primates because of the background of information available on this species relating to ovulation and semen collection. Semen was collected by electroejaculation and the seminal coagulum was incubated to obtain a sperm suspension for distribution to culture/slide compartments. The culture medium (TC-199 or Ham's F-10) included 20% agamma calf serum. Follicular growth was stimulated in females by a regime of 1 mg FSH daily for 4 days followed by 500 iu. HCG, i.m. During the summer months FSH administration was extended to 5 days. Oocytes were recovered for culture 8 to 12 hours after HCG administration by follicular aspiration techniques employing either laparotomy or laparoscopy. The oocyte along with the associated cumulus mass and follicular fluid were distributed to culture/slide chambers according to their follicular sources. The ova in culture were observed at intervals for evidence of fertilization. At varying stages following fertilization the ova were removed from

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[1]Presented at the 8th Ann. Mtg. of the Soc. for Study of Reprod., Fort Collins, Colorado, July 22-25, 1975.

culture and transferred to the oviducts of synchronized recipient females using a laparoscopic technique. The recipient females with transferred embryos were then monitored for pregnancy and gestational changes. Oocytes recovered from follicles of gonadotropin-treated females and cultured *in vitro* with spermatozoa resulted in fertilized ova capable of developing to the eight-cell stage in culture. In total 559 follicular oocytes were recovered from 1617 follicles for a 34.6% average recovery. Of these ova 19.7% were atretic at the beginning of the culture period. Of the remaining 449 ova, 34.7% matured to the metaphase II stage and 51 of these 156 mature ova were fertilized *in vitro*. Ova retaining cumulus and corona radiata vestments showed a higher tendency to mature (62% maturing) when compared to ova without such vestments (7% maturing). Two of 3 females receiving embryo transfers at the 2-cell stage had an enlarged uterus 60 days after transfers. However, at 90 days the females were nonpregnant.

IN VITRO FERTILIZATION OF NONHUMAN PRIMATES[1]

W. R. Dukelow and T. J. Kuehl
Endocrine Research Unit, Michigan State University
East Lansing, Michigan 48824

A total of 604 oocytes from 1754 squirrel monkey ovarian follicles were recovered (34.4%) for use in *in vitro* fertilization trials. Of these, 119 (19.7%) were atretic. Of the remainder, 36.1% matured to the metaphase II stage and 58 (33.1%) were fertilized *in vitro*. Cleavage was noted to the eight-cell stage. Two-cell ova were transferred to the fimbria of recipient females with two animals showing preliminary signs of pregnancy but failing to complete pregnancy. All procedures of ovum recovery and transfer of two-cell embryos were done laparoscopically to avoid major surgery with the animals.

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[1]Presented at the Colloquium of the French Nat. Soc. for the Study of Sterility and Fertility, Paris, France, December 11, 1975.

VITA

Name:	THOMAS JOHN KUEHL
Born:	February 7, 1950
Birthplace:	Marshfield, Wisconsin
Formal Education:	Rockford West High School Rockford, Illinois
	Michigan State University East Lansing, Michigan
Degrees Received:	Bachelor of Science (with honors) Michigan State University, 1972
	Master of Science Michigan State University, 1974
Member of:	International Primatological Society
	American Association for the Advancement of Science
Honors:	Recipient of the Sigma Xi Award for Outstanding Graduate Research, 1975.