IN VITRO FERTILIZATION IN SAIMIRI SCIUREUS

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ABSTRACT

IN VITRO FERTILIZATION IN *SAIMIRI SCIUREUS*

By

Thomas John Kuehl

A restraint device was constructed to allow a technician to capture, restrain, and collect semen from a squirrel monkey (*Saimiri sciureus*) without assistance. Male squirrel monkeys are effectively restrained using this device with very little danger of biting the handler or causing injury to the animal during the electroejaculation procedure.

Captive squirrel monkeys show a seasonal response to a subtle ovulation induction regime of 5 days of progesterone, 4 days FSH (1 mg/day) 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 or 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 ovulation while increasing HCG was ineffective.

An *in vitro* fertilization system was developed for *S. sciureus*. Adult females were induced to ovulate without regard to natural cycle.

Laparotomies were conducted 4 to 12 hours after HCG administration, and follicular oocytes were recovered in culture medium. A solution of F 10 medium supplemented with 20% agamma newborn calf serum worked best. Semen ejaculates were mixed in the medium and the sperm suspension and oocytes were cultured at 37°C in 5% CO_2 in an air environment. A culture chamber slide was found to offer good visibility of fertilization as well as development of the oocyte to 4-celled embryos. Of 719 follicles aspirated, 176 oocytes were recovered. Fifty-nine developed to the first polar body stage and 20 of the 59 were fertilized (34%). Twelve of the 20 fertilized ova reached the 2-polar body, 2-pronucleate stage and the rest were two or more cells (7 embryos reaching the 4 cell stage).

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By

Thomas John Kuehl

A THESIS

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To all my friends,

thank you for being kind.

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To the greatest Love in my life, no words are meaningful and precious enough to express thanks and appreciation, only love (LITLTLTWAWWTPOL!).

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INTRODUCTION

In vitro fertilization has been accomplished in a wide variety of non-primate mammalian species including the rabbit, rat, cat, mouse, and guinea pig as well as in at least one primate species, man. Studies in nonhuman primates have brought forth few *in vitro* fertilized ova. For example, one laboratory which has reported *in vitro* fertilization for rabbits and humans has tried unsuccessfully to develop a Rhesus monkey *in vitro* fertilization system (Brackett, personal communication). Kraemer has obtained a single *in vitro* fertilized baboon embryo, but has yet to duplicate his effort (Kraemer, personal communication). An experimental system for *in vitro* fertilization of nonhuman primate oocytes would allow experimental manipulation of a variety of factors, leading to a better understanding of primate fertilization and of the potentials for control of genetic abnormalities.

In vitro fertilization can be a useful technique for studies of the time sequence of morphological and biochemical events involved in sperm penetration and fertilization of an ovum and the subsequent development of the embryo. Future applications of this technique might include studies involved with:

1. Defining the physical and chemical conditions that allow fertilization

2. Sperm capacitation (the normal process whereby the sperm attain the capability to fertilize an ovum)
3. Gamete aging
4. The effects of chemical (i.e., teratogenic) substances on fertilization and early genetic development of the embryo
5. Evaluation of the anticipated incidence of genetic or gross morphologic abnormalities under varying conditions of the fertilization and early embryonic environment.

Another possible application of *in vitro* fertilization includes (with embryo transfer techniques) reducing generation time for certain strains of nonhuman primates. In biomedical research these procedures may be a means of circumventing some types of human infertility in which a functionally intact ovary and uterus are present. However, successful embryonic transfers have yet to be performed in primates.

Saimiri sciureus has several advantages for use in studies of nonhuman primate reproductive physiology. They are relatively inexpensive to purchase and maintain, and they are readily available at the present time. They are smaller, require simpler caging and are less susceptible to tuberculosis than the old world monkeys. Of particular importance to *in vitro* fertilization work are the acrocentric-to-submetacentric chromasomal ratio differences found in karyotypes of squirrel monkeys from different geographic regions. These differences will better enable observation of genetic manipulations. One disadvantage, of the use of *S. sciureus* as donors of oocytes for *in vitro* fertilization and as recipients of fertilized embryos, is their seasonal

response to ovulation induction regimes, which creates an "off" season for studies.

The objectives of these investigations were:

1. To develop a technique for restraint of *S. sciureus* males for electroejaculation,
2. To develop an ovulation induction regime for *S. sciureus* females which will be effective during the three month season of low ovulatory response,
3. To define the conditions necessary for the *in vitro* fertilization of *S. sciureus* ova which allows development of the zygote to at least the four-cell stage.

A RESTRAINING DEVICE FOR ELECTROEJACULATION OF SQUIRREL MONKEYS *(SAIMIRI SCIUREUS)*

The following is a paper as will appear in the April, 1974 issue of *Laboratory Animal Science*.

Summary: A simple restraint device is described for the squirrel monkey which allows a single operator to collect semen samples from unanesthetized animals.

Two methods of electroejaculation have been used for semen collection in nonhuman primates. The first, penile stimulation has been primarily used in macaques (Mastroianni and Manson, 1963; Valerio *et al.*, 1969); whereas rectal stimulation has been utilized in species ranging in size from the tree shrew (*Tupaia glis*) to the chimpanzee (*Pan troglodytes*) (Fussell, Roussel and Austin, 1968; Arvis, Cohen and Augros, 1970; Weisbroth and Young, 1965). Generally such procedures have involved phencyclidine sedation (Bennett, 1967; Kraemer and Vera Cruz, 1969), although some investigators, using squirrel monkeys have relied on manual restraint by a gloved assistant (Roussel and Austin, 1968).

The present study was designed to provide a device and technique whereby a researcher could capture, restrain, and collect semen from a squirrel monkey without assistance.

MATERIALS AND METHODS

Restraining Device

The basic design was modified from a 40° angled reclining trough described and depicted by Lang (1968). The device is made by joining two 18" X 4 1/2" X 3/4" pieces of plywood in the form of a V. At one end of the V, the corner is cut away to allow rectal probe access to the animal's tail and rectum. A 5" X 4" x 3/4" piece of plywood was cut in half along a 45° angle to provide matched supportive legs. These were then attached to the restraint device as shown in figure 1. An 8" X 6" X 2 1/2" sponge is attached to a 7 3/4" X 6" X 3/4" plywood board, to be placed over the arms and abdomen of the animal. Screw eyes were used to attach the cotton cords used for leg restraints and to fasten the sponge-padded board to the trough. The trough was covered with cloth and any exposed corners taped or covered to prevent harm to the monkeys. The completely assembled device is shown in figures 1 and 2.

Technique for Use

When the animal is placed in the device care must be taken so that the handler and monkey are not harmed. The handler should use a minimum of manual force and wear primate handling gloves. With the squirrel monkey held in the left hand, the monkey's forelimbs and body are grasped in the right, from the front of the animal. The monkey is then placed down into the device face up (fig. 3). The sponge-padded board is placed over the monkey with the left hand while the handler slowly withdraws his right hand (fig. 4). Care is taken so the animal

does not get his hands free or twisted. Care must be taken when securing this board into position (fig. 5), so that the animal's breathing is natural and not labored. The thickness of the sponge and the dimensions of the padded board prevent such restriction. With the monkey restrained, the legs can now be secured with cotton cord (fig. 6). The device is then set on its side (fig. 7) to allow electroejaculation. In this position the researcher can position the rectal probe and manage the power-supply unit to obtain a semen sample by previously reported techniques (Fussell, Roussel and Austin, 1967; Roussel and Austin, 1968; Bennett, 1967) (fig. 8).

After the procedure has been completed, the monkey's rear legs are released. The monkey can be freed in a cage by reversing the procedure for putting it in the device.

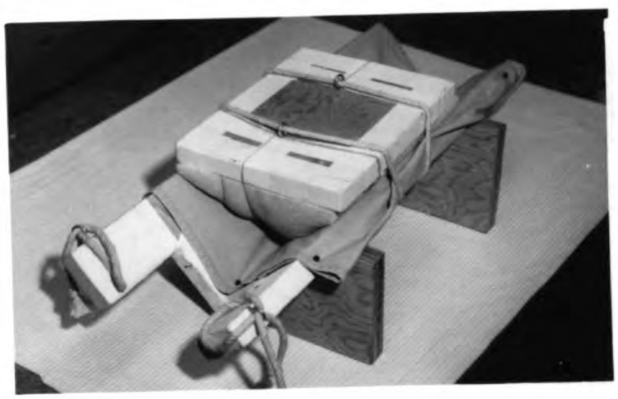
RESULTS AND DISCUSSION

The described device has been repeatedly used in this laboratory for the twice weekly collection of semen from all colony males.

Male squirrel monkeys are effectively restrained using this device with limited danger of biting the handler and with no danger of being injured during the electroejaculation procedure. The animals are readily ejaculated with only the momentary discomfort of the technique. Only one person is required for the entire procedure using this device, whereas formerly two individuals were needed.

Although used only with the squirrel monkeys, slight adaptation of size, would allow the use of the restrainer for a variety of nonhuman

primates up to 2 kg in weight. Similarly, although wood materials were used in this construction, other materials, such as acrylic plastics, could be used. The device can also be used to restrain monkeys for other procedures such as collecting blood samples, oral infusions, etc.



Figs. 1 and 2. The assembled restraint device.

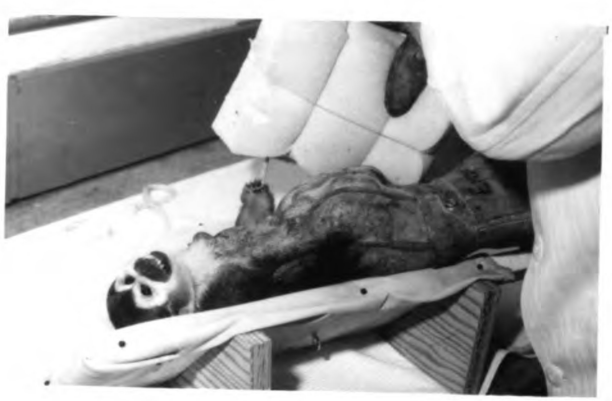


Fig. 3. Positioning of a male squirrel monkey in the device.

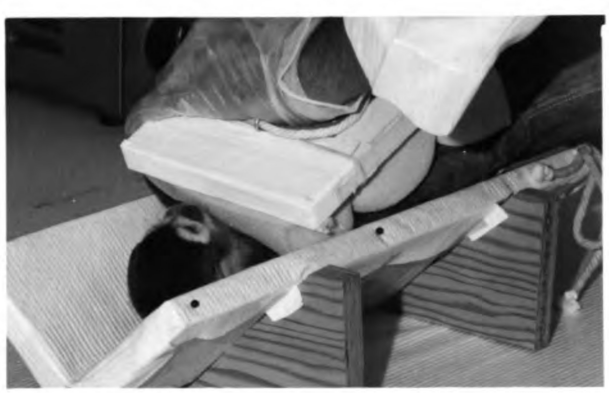


Fig. 4. Placement of the padded board into position.

Fig. 5. Placement of the padded board into position.

Fig. 6. Fastening of the legs.

Fig. 7. Positioning the device for the experiment.

Fig. 8. Positioning the rectal probe for electroejaculation.

OVULATION INDUCTION IN *SAIMIRI SCIUREUS* DURING THE SEASON OF LOW OVULATORY RESPONSE

This study was undertaken to determine if modifications of a presently used subtle ovulation induction procedure could increase its effectiveness for use during a season of low ovulatory response.

Literature Review

The first reported efforts to induce ovulation in *Saimiri sciureus* were by Bennett (1967a). He used pregnant mare's serum (PMS) and human chorionic gonadotropin (HCG) to induce superovulation. He also reported fertilization (but not pregnancies) of superovulated ova by artificial insemination. He suggested that concentrations of PMS may have accelerated ovum transport through the oviducts by increasing systemic levels of estrogen from the ovary (Bennett, 1967b).

In 1970 Dukelow proposed a hormone regime to control the time of ovulation and to limit the number of ovulations per animal to one or two. The basic technique involved administration of progesterone for five days, to mimic the luteal phase of the cycle, followed by four days of follicle stimulating hormone (FSH) and a single injection of HCG (Dukelow, 1970a). This ovulation induction regime was repeatable and used in studies of a contraceptive steroid, megestrol acetate (Harrison and Dukelow, 1971).

Ovulation has been induced in immature squirrel monkeys with PMS and HCG (Fajer and Bechini, 1971). Extensive follicular growth was noted, but no time course for this growth was indicated. Ovulation was followed by luteinization detected histologically. Detectable amounts of pregnenolone and progesterone were found in ovarian venous blood two days after ovulation. By five days post ovulation less than half of the animals still had elevated hormone levels. The interstitial tissue was suggested as a source of the pregnenolone.

Recently, induction of ovulation has been reported in *S. sciureus* using three daily injections of progesterone and a single injection of PMS to reinforce a cycle based on periodic changes in vaginal cytology (Cline, 1972; Gould, Cline and Williams, 1973). The timing of the injections was determined after ascertaining the percentage of cornified cells in the vaginal smear and by giving the progesterone at the lowest percentages. Progesterone was used to synchronize several females and initiate timing to the PMS injection. The PMS stimulated follicular maturation leading to ovulation. Of the twelve animals used in this study (Gould, Cline, and Williams, 1973), only six were reported to have exhibited regular eight day cycles. The remaining animals either showed irregular cycles or no cycles at all. Travis (1973) has reported seasonal estrous cycles alternating with periods of sustained anestrus and no apparent seasonal triggering stimulus in squirrel monkeys. These seasonal anestrus periods then limit the applicability of the natural cycle reinforcing ovulation induction regime. A summary of published induction schemes and the results are shown in tables 1 and 2.

Table 1. Ovulation induction by PMS and HCG in *Saimiri sciureus*

Dose of PMS (i.u.)	Dose of HCG (i.u.)						
	500 2X daily days 5-9	250 2X daily days 5-9	250 2X daily days 6-9	500 days 6-9	500 to 250 eve day 9	45 hrs + post PMS	44 hrs post PMS
200 (twice daily) days 1-9	[a] 1/1 (100) 14	[a] 1/1 (100) 2*		[c] 4/4 (100) 4.8			
200 days 6-9[1]					[c] 3/12 (25) 1.0		
50 days 1-9			[d] 8/16 (50) **				
20 (twice daily) days 1-9	[a] 1/1 (100) 7	[a] 2/2 (100) 4	[b] 3/3 (100) 7				
10 (twice daily) days 1-9		[a] 1/1 (100) 5					
100 day 6[2]						[e] 12/16 (75) 1.1	[e] 0.6 (0) 0
2-5 (twice daily) days 1-9	[a] 0/1 (0) 0	[a] 0/2 (0) 0					
No PMS[1]					[c] 0/6 (0) 0		

Key: X (Y)
Z

X = No. ovulating/No. treated
Y = % ovulations
Z = Mean no. ovulations per animal ovulating

*One ovary was cystic.

**Animals immature sexually 12-15 months old.

[1] 5 mg progesterone/day on days 1-5.

[2] 5 mg progesterone/day on days 1-3 (with day 1 being the day of an animal's cycle with the lowest percentage of cornified cells in the vaginal smear).

[a] Bennett, 1967a.

[b] Bennett, 1967b.

[c] Dukelow, 1970a.

[d] Fajer and Bechini, 1971.

[e] Cline, 1972.

Table 2. *Saimiri sciureus* ovulation induction methods

Follicle stimulating treatment*	Dose of HCG 250 i.u. eve of day 9			Dose of HCG 250 i.u. eve of day 9		
	No. ovulating / No. treated	(% ovulating)	Mean no. of ovulations	No. ovulating / No. treated	(% ovulating	Mean no. of ovulations
100 i.u. HMG** days 6-9	[a] 1/5	20	1.0			
1 mg FSH days 6-9	[a] 13/25	52	1.2	[a] 8/15	27	1.3
1 mg FSH days 6-9	[b] 53/108	49	1.4			

*All animals received 5 mg progesterone/day on days 1-5.

**Assayed as equivalent to 75 i.u. FSH and 25 i.u. LH (Dukelow, 1970b).

[a] Dukelow, 1970b.

[b] Dukelow, Harrison, Rawson and Johnson, 1972.

More recently the effectiveness of the regime proposed by Dukelow (1970a) was correlated with the length of time in captivity and by the season of the year (Harrison and Dukelow, 1974a). These studies demonstrated a peak response to the ovulation regime from December through April with a minimal response in July, August and September. The animals appear to have required at least nine months to acclimate to the laboratory environment.

Materials and Methods

Animals

S. sciureus, all of the Brazilian type (export point of origin: Leticia, Columbia), were purchased from Tarpon Zoo, Inc., Tarpon Springs, Florida. The 30 animals, which were used, had been in captivity for 1.75 to 2.75 years at the start of the experiment. These squirrel monkeys were from the same group studied by Harrison and Dukelow (1974a). The monkeys were maintained in a large gang cage, which is 3.3 meters long, 2.5 meters high and 1.3 meters wide. This cage contained numerous perches to allow for greater mobility and social interaction, while multiple feeding and watering stations were provided to allow easy access to food and water. During the periods that the animals were being tested on an ovulation regime, they were held in stainless steel, flush type cages, 0.6 m X 0.7 m X 0.8 m. They were fed commercial monkey feed (Wayne Monkey Diet, Allied Mills) and received fresh water *ad libidum*. All cages were washed daily. The animal room had fluorescent lighting on a 12:12 light-dark cycle. The temperature was maintained at 21° ± 2°. Relative humidity was not controlled.

Ovulation Induction Procedures

Squirrel monkeys in captivity show a seasonal response to a subtle ovulation induction regime consisting of five days of progesterone (5 mg), four days of FSH (1 mg) and an ovulatory dose of HCG (500 i.u.) (Dukelow, 1970). Ovulation occurs 60% of the time except during July, August and September when the effectiveness drops to zero (Harrison and Dukelow, 1974a). This seasonal response to the regime may be due to its design to produce only single or double ovulations. This subtle regime may be only a minimal stimulus. If the normal endogenous gonadotropin levels drop during the summer, then the minimal scheme could become subminimal. To test this hypothesis 18 adult female *S. sciureus* were randomly allotted to a 3 X 3 factorial design with two variables of HCG ovulatory dose and FSH treatment as follows: (1) HCG (A.P.L., Ayerst Laboratories, New York), either 500, 1000 or 1500 i.u. given intramuscularly in 0.25 ml of diluent, and (2) FSH (FSH-P, Armour-Galdwin Laboratories, Omaha, Nebraska), either (a) 1 mg FSH for 4 days, (b) 1 mg FSH for 5 days, or (c) 2 mg FSH for 4 days given intramuscularly in 0.2 ml of diluent. All treatments were preceded by the standardizing dosage of 5 mg progesterone (Δ4-pregnen-3,20-dione, Sigma Chemical Company, St. Louis, Missouri) in 0.25 ml cottonseed oil given subcutaneously for 5 days. This amount of progesterone was the same dosage that was used by Dukelow (1970).

An alternate hypothesis of the summer anovulatory period could relate to the need for endogenous estrogens or estrogen cyclicity in order for ovulation to be induced. In other words, low levels of

estrogen (perhaps due to a decreased ovarian sensitivity to gonadotropins) or the lack of a changing estrogen-progesterone ratio may account for the ineffectiveness during the summer. This was tested in a two phase experiment involving 12 animals. In phase one a group of 6 animals was tested using 20 µg of estradiol (1, 3, 5, 10)-estrotrien-3, 17B-diol, Mann Research Laboratories, New York, New York) in oil administered subcutaneously for four consecutive days and then followed by treatment with the standard ovulation induction regime as described earlier. In the second phase of the experiment a changing ratio of estradiol (E) and progesterone (P) over a ten day period was used. On the first two days 20 µg E was given. On days three and four, 15 µg E and 1.25 mg P; days five and six, 10 µg E and 2.5 mg P; days seven and eight, 5 µg E and 3.75 mg P; and on days nine and ten, 5.0 mg P. All daily dosages were given in 0.25 ml oil subcutaneously. This regime was followed by four days of FSH (1 mg) and an ovulatory dose of 500 i.u. HCG. The estradiol doses were based on those reported by Graham (1969) who determined the doses of estrogens necessary to cause proliferation of the squamous epithelium of the vagina and cervix of ovariectomized squirrel monkeys.

Laparoscopic Examination of the Ovaries

The ovaries of the ovulation-induced squirrel monkeys were examined by laparoscopy 12 and 36 hours after HCG injection to ascertain if ovulation had occurred. The laparoscopic procedures have been previously described (Dukelow, Jarosz, Jewett and Harrison, 1971; Harrison and Dukelow, 1974b) for a variety of species including *S. sciureus*.

Briefly the procedure used was as follows: The monkeys were anesthetized with 16.2 mg of sodium pentabarbital (Halatal, Jensen-Salsbery Laboratory, Kansas City, Missouri) administered intraperitoneally. The animals were then prepared by clipping the hair from the abdominal region and cleaning the skin surface with zephiran chloride. The animals were placed, head down, on an examination table tilted at 30°. A heating pad was provided to prevent anesthetic hypothermia. A midline incision, one centimeter in length, was made through the skin in the area of the umbilicus. A trocar-cannula was inserted in the incision and through the muscle layer into the abdominal cavity. The trocar was removed and replaced with the laparoscope. The abdomen was insufflated with humid 5% CO_2 in air. A Verres cannula was inserted into the peritoneal cavity approximately 2 cm posterior and lateral to the laparoscope incision. This probe was used to manipulate internal organs to obtain a better view of the ovaries.

Following the examination the incision was closed by suturing the skin with a chronic 3-0 suture. Nitrofurazone powder (Furacin, Eaton Laboratories, Norwich Pharmacal Co., Norwich, New York) was placed on the incisions and then covered with nitrofurazone ointment. Each monkey also received 150,000 units of procaine penicillin G intramuscularly.

Experimental Design

The ovulation induction regimes tested have been described in a previous section on ovulation induction methods. Two types of variations were employed in the design of ovulation induction methods that might prove effective in the summer (estrogen pretreatments and increased gonadotropin doses). Animals were assigned to groups at random with the single condition that one-half of the animals in each group had ovulated to a control regime (Dukelow, 1970) administered in June, while the other half had not. Those animals that ovulated in June were considered sensitive to the standard induction regime. The 3 X 3 factorial design was evaluated using analysis of variance to test for significant variance in ovulatory responses due to recognized sources of variation. The ovulatory response was defined as the number of ovulation points viewed per ovary at the 36 hour laparoscopic observation. The mean of this measure for any group takes into account both the proportion of animals ovulating and the number of ovulations of each animal. Recognized sources of variation were treatments (FSH treatments, HCG doses, and interaction between the hormones), replications (months that the regimes were tested), June sensitivity, and the ovaries of the animal (right and left). If significance was found for a source of variation, the Student Newman Keuls test was used to compare the mean responses. In all statistical analysis a probability of less than 0.05 that the results could have occurred by chance was accepted as significant.

Results

The results of the various FSH and HCG treatments are shown in table 3. The table indicates the ratio of squirrel monkeys ovulating to the total number of animals tested. Both an increase in duration and dose level of FSH administration increased the proportion of animals ovulating, while increasing dosages of HCG were less effective. The animals given the control treatment did not ovulate throughout the summer trial period, while those animals given twice the control dosages of FSH and HCG showed the highest incidence of ovulation. The ovulatory response was determined for each of the animals and analyzed. The results are shown in table 4. The analysis indicates that there was an effect on the ovulatory response due to treatment with the various gonadotropins. Furthermore, there were differences among HCG levels used and FSH treatments, but no significant interactions. Treatment with FSH (1 mg) for 5 days gave a significantly higher ovulatory response than either of the other two FSH treatments whereas 1000 i.u. of HCG showed the highest measure of ovulatory response of the three HCG levels tested. There was no significant difference in the ovulatory responses between months or replications of the treatments. Right ovaries showed a significantly higher ovulatory response than left ovaries. Animals ovulating to the standard (control) regime in June showed a significantly higher ovulatory response than those that had not ovulated in the June trial.

Table 3. *Saimiri sciureus* ovulation in response to varying levels of HCG and FSH

FSH dosage and duration	HCG dosage (i.u.) 500 Ovulation ratio	500 Ovulation per animal ovulating	1000 Ovulation ratio	1000 Ovulation per animal ovulating	1500 Ovulation ratio	1500 Ovulation per animal ovulating
1 mg/day for 4 days	0/8[a]	0.0	2/8	1.5	0/8	0.0
1 mg/day for 5 days	4/8	1.0	4/8	2.5	4/8	1.0
2 mg/day for 4 days	3/8	1.0	5/8	1.0	0/8	0.0

[a]Control group.

Table 4. Analysis of variance table of ovulatory response[a] of *Saimiri sciureus* to varying levels of FSH and HCG

Source	df	SS	MS	F ratio
Treatment	8	5.10	0.64	3.56*
HCG	2	2.26	1.13	6.28*
FSH	2	2.43	1.22	6.78*
HCG X FSH	4	0.41	0.10	NS
Months	3	0.19	0.06	NS
Side of ovary	1	1.17	1.17	6.50*
June sensitivity	1	1.56	1.56	8.67*
Error	130	23.14	0.18	
Total	143	31.16		

*Significant at $P < 0.05$.

[a]Ovulatory response measured as number of ovulation points per ovary.

The results of the estrogen-progesterone pretreatment trials are shown in table 5. Although there was no difference between the two types of pretreatment regimes in terms of the ratio of animals ovulating per total tested, all the ovulations attributed to the separated sequential regime of estradiol and progesterone pretreatment were multiple as opposed to the single ovulations of the pretreatment regime. Only 4 of 18 animals used in the FSH-HCG trials did not ovulate during the study period. Two of these animals ovulated in subsequent experiments. Of all the animals used only 2 of 30 have not ovulated in response to any regime in captivity. At 12 hour laparoscopic examinations 35 ovulation points were detected throughout the study period. Forty-three percent of these were on the left ovaries and 57 percent on the right. By the 36 hour examination 23 more ovulation points were detected with only 26% on the left ovary and the remaining 74% on the right ovary.

Table 5. *Saimiri sciureus* ovulation induced in two estradiol progesterone pretreatments followed by FSH-HCG

Pretreatment scheme	Ovulation ratio[a]		Ovulation per animal ovulating
Separated sequential estradiol and progesterone pretreatments	*4/18	(22.4%)	2.8
Estradiol and progesterone given in combinations of changing proportions	3/18	(16.7%)	1.0

*All ovulations multiple.

[a]Data given as ratio of number of animals ovulating to total number of animals tested.

Discussion

The goal of these studies was to develop an ovulation induction scheme for *S. sciureus* that would be effective during the season of low ovulatory response. Two hypotheses as to why this seasonal response occurred were suggested and several ovulation induction regimes were designed on the basis of these theories. One theory explained the low ovulatory response observed in the summer was due to decreased amplitudes of cycling estrogen and progesterone levels. The other theory attributes the low ovulatory response to decreased gonadotropin levels. However, until hormone assay data is available for *S. sciureus* these theories can only be speculative.

The first theory was that the standard ovulation regime required estrogen-progesterone cyclicity and that the season of low ovulatory response represents a period where this endogenous cyclicity is reduced. The test treatments involved administration of estradiol followed by progesterone or estradiol and progesterone in a gradually changing ratio. The results indicate that while these treatments are effective at producing some response, the proportion of animals ovulating is still low. The only difference between the treatments was that estradiol and progesterone, given separately, produced all multiple ovulations with an average of 2.8 ovulations per animal ovulating. This finding may be of significance when one considers that Bennett (1967b) found superovulation to be related to a high rate of tubal transport of ova. If the ovulation induction scheme is to induce ovulation to allow artificial insemination, natural mating, or synchronization of a recipient for

embryo transfer, regimes which result in the ovulation of more than two ova or abnormal rates of tubal transfer should be avoided. Perhaps increasing the amounts of estradiol administered would increase the proportion of animals ovulating in response to the test regimes. The estradiol dosage chosen was adapted from work by Graham (1969) involving estrogen-induced changes at the cervical squamo-columnar junction of ovariectomized squirrel monkeys. He found that 2.3 μg of estradiol benzoate per day for 8 days was sufficient to produce signs of estrogenic stimulation of the genital organs. The pretreatments in the present studies used estradiol in the dosages previously described and represent lower total estrogenic activity than those used by Graham.

The alternative hypothesis was that the standard ovulation induction regime, which had been designed to produce only single or double ovulations, employed only minimal exogenous gonadotropic stimulation. The season of low response then might represent a period when endogenous gonadotropin levels are low. Thus the minimal regime becomes subminimal. To counteract the proposed decline in endogenous gonadotropins, 3 X 3 factorial design was used to test three levels of HCG and three levels of FSH. An increase in the proportion of animals ovulating was noted with either increased duration of FSH administration or increased dosage of FSH over controls. Increasing levels of HCG from the control level of 500 i.u. were less effective. This could indicate that the season of low response may be due to a decrease in endogenous follicle stimulating gonadotropin levels or to a decrease in ovarian sensitivity to exogenous follicle stimulating hormone.

The analysis of variance in the ovulatory response, defined as the mean number of ovulation points per animal observed by 36 hours after HCG injection, provide further insight into the effects of gonadotropic stimulation. No significant variation occurred between the replicates of the design indicating that little or no refractoriness developed to the FSH and HCG at the levels and durations tested. The animals that were sensitive to the standard regime administered in June also showed a higher ovulatory response during the study period justifying the rationale that sensitive and nonsensitive animals should be equally apportioned to all experimental groups. The results also indicate that the right ovaries of the animals gave a higher ovulatory response than the left ovaries. Furthermore, data was provided that indicate this variation in response occurred between 12 and 36 hours after HCG injections. A possible explanation for this variation between ovaries suggests squirrel monkeys could be predisposed to ovulate more from the right ovary than from the left.

Rawson and Dukelow (1973) found only a slight difference in the proportion of ovulations from the ovaries of normally cycling *Macaca fascicularis* (48.7% from the left and 51.3% from the right). Harrison (1973) found slight favoring of the left ovary in the same population of *S. sciureus* used in the present study (51.4% ovulations on the left and 48.6% on the right). However, Harrison's data was derived from total observations and made no distinction between observations made at various times following HCG injection. Since the investigators cited above also used the laparoscope for their ovarian examinations, it is

unlikely that the procedure *per se* produces any marked favoring of one ovary. Wallach (1973), in discussing a possible correlation between cycle length and side of ovulation with respect to the previous cycle in Rhesus monkeys, cites the mountain viscacha (a South American rodent) as showing a predominance of right-sided ovulations. However, both ovaries appear to have an equal potential to function. It is possible that squirrel monkeys naturally ovulate more frequently from the right ovary. Wallach (1973) speculates that ovulations which occur on the same ovary as in the previous cycle will be delayed, at least in the Rhesus monkey, due to an inhibitory action of local (ovarian) progesterone on developing follicles. The source of this progesterone would most likely be luteal tissue in the ovary. There may be a possibility that such local progesterone levels in the right ovary of a squirrel monkey would tend to delay ovulation. That is, if the animal is induced to ovulate when sufficient progesterone levels are present in the ovary. This could explain an increase in right ovarian ovulation points with time after HCG, but more work is needed to verify this hypothesis.

The results of the analysis of the ovulatory response indicate that the treatment with the highest response was 5 days of FSH and 1000 i.u. of HCG. This regime, however, has the distinction of producing 2.5 ovulations per animal ovulating and may not be desirable for the reasons previously mentioned. By using the least amount of hormone and still getting 50% of the animals to ovulate, the regime which uses 5 days of FSH (1 mg per day) and 500 i.u. of HCG seems the most promising. This study does, however, show that many possible regimes of slightly

increased gonadotropin dosages will increase the number of ovulations per animal and, more importantly, increase the number of animals ovulating during the low response season.

IN VITRO FERTILIZATION EXPERIMENTS IN *SAIMIRI SCIUREUS*

Literature Review

Studies of *In Vitro* Fertilization in Human Primates

Several reviews have been published in recent years documenting the continued progress of research on human oocyte maturation and fertilization *in vitro* leading to work on human embryo transfer (Thibault, 1969; Brackett, 1969; Brackett, Seitz and Mastroianni, 1971; Kennedy, 1972; Cline, 1972a; Edwards, 1973). In light of these numerous reviews, this review will briefly overview the major works already reviewed and update the more recently published accounts of *in vitro* fertilization in primates.

Many of the early accounts of *in vitro* fertilization of human ova 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. The studies of human *in vitro* fertilization may be divided into two categories, such as has been done by Kennedy (1972): those which fertilized oocytes after their meiotic maturation *in vitro* and those which fertilized *in vivo* matured ova. Studies of the former type were first reported by Menkin and Rock (1944, 1948). They observed over 800 oocytes and attempted to fertilize

138. After culturing the oocytes for 27 hours then adding washed spermatozoa, they obtained 2 two-celled and 2 three-celled embryos. The stage of maturation of the oocytes at the time of insemination was not recorded. Doubt is 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.

Shettles studied over 1,000 oocytes (1953, 1955). About 200 were subjected to insemination by fresh human semen and cultured in a medium of follicular fluid and bits of tubal mucosa. 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 place some doubt on these studies as well.

Petrucci reported human *in vitro* fertilization, but signs of maturation and incidence of either successful culture, fertilization or parthenogenesis were not given (1961). This work has never been published in an acknowledged scientific journal.

Hayashi observed fertilization of 20 of 160 follicular oocytes he cultured *in vitro* in media with human serum proteins, basal salt solution and hormones (1963). 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.

Edwards, Banister and Steptoe (1969) reported fertilization by culturing follicular oocytes 35 hours in a variety of media containing follicular fluid, then inseminating the cultures with ejaculated, washed spermatozoa and monitoring for signs of formation of pronuclei. No pronuclei were seen in control ova, but 11 pronucleate eggs were found in inseminated cultures. However, 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 human *in vitro* fertilization.

Several investigators have 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 1969 Jacobson, Sites and Arias-Bernal reported *in vitro* maturation and fertilization of human follicular oocytes. They cultured oocytes in a medium of Hanks 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. Eight of 11 were female embryos (46XX) and three were male embryos (46XY). All had cleaved to at least the four-cell stage.

Seitz, Rocha, Brackett and Mastroianni (1971) used human ova matured *in vitro* for 24 hours to obtain fertilization in 8 of 50 ova inseminated with human spermatozoa preincubated in a Rhesus monkey uterus. They used a medium of F-10, heated human serum and sodium

estrone sulfate (Premarin). None of the twenty control ova cleaved. The fertilized embryos ranged from a 2-celled stage seen at 40 hours after insemination to a 12-celled stage at 72 hours after insemination. Blastomeres were demonstrated to contain chromatin material using lacmoid stain.

In 1968, Jagiello, Karnicki and Ryan collected oocytes from gonadotropin pretreated women (pituitary follicle stimulating hormone (FHS) 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. Steptoe and Edwards (1970) recovered oocytes by laparoscopy from pre-ovulatory follicles of women treated with human menopausal gonadotropin (HMG). They also found *in vivo* matured oocytes. Based on these studies, Edwards, Steptoe and Purdy (1970) used HMG and HCG to stimulate *in vivo* maturation of oocytes in humans and then recovered the oocytes by laparoscopy 30 to 32 hours after HCG injection. In this manner 393 oocytes were obtained for culture. The oocytes were cultured 1 to 4 hours before insemination and examined for signs of fertilization or cleavage 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 effect the sperm acrosome.

Recently Soupart and Morgenstern (1973) have obtained *in vitro* penetration by sperm through the zona pellucida and fertilization of oocytes that were recovered from ovaries of gonadotropin-treated women and matured *in vitro*. These investigators 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 gonadotropins (FSH, LH and HCG). The oocytes were next transferred to a fertilization medium made up of a modified Bavister's medium, washed spermatozoa, and 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 which seems low when compared to the same type of maturation and length of culture by other investigators. The authors found that gonadotropins in the fertilization media did significantly increase sperm penetration into or through the zona pellucida; however, more oocytes showed sperm penetration than matured. The authors concluded that sperm penetration is not dependent on oocyte maturation but may require sperm capacitation. The sperm penetration enhancement was thought to be mediated by the gonadotropin stimulated follicle cells present around the oocytes in the fertilization medium. The authors also observed that the fertilizing sperm tail or its remnants could not be found in oocytes recovered after 28 hours of fertilization culture, either in the fresh state or

after fixing and staining. Bavister, Edwards and Steptoe (1969) have previously reported observing the midpiece and the tail of spermatozoa in human fertilized ova 11 to 14.5 hours after insemination of oocyte cultures with washed sperm. Soupart and Strong (1974) have shown the presence of bits of degenerating sperm tail in oocytes of the late pronucleate stage, but these were only visible by means of electron microscopy. They discussed evidence that human sperm require capacitation and that it can take place during contact with follicular cells in the culture medium. Soupart and Strong were not able to get 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 is 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.

The most recent development in studies of the progression from oocyte to transplanted embryo was reported as a case study of Shettles in November, 1973. An oocyte was aspirated from a woman undergoing surgery to correct a defective oviduct. The oocyte was cultured until the first polar body appeared, then follicular fluid, tubal mucosa and spermatozoa were added. The culture environment was regulated with respect to oxygen tension, pH and temperature (37°C). Shettles suggests that addition of human placental serum to the medium may increase success. The matured oocyte was fertilized and cultured five days to the

blastocyst stage. The embryo was then transferred via a pipette through the cervix to the uterus of a second woman. The menstrual cycles of both women had been hormonally synchronized. Two days after transfer, the uterus of the recipient was examined following hysterectomy. The blastocyst was located in the upper, posterior lining of the uterus. The investigator reported that the embryo was several hundred cells and was properly nidating. As of yet, no embryo transfer has been reported in primates which has led to the birth of a live infant.

There are several reasons to doubt the validity of the interpretation of the transfer mentioned. First, the single experiment seemingly conquered all previous problems which have baffled scientists for several years. Secondly, the information was published without detail as a discussion following a presentation of another scientist and the material has not been submitted for formal review and publication. Finally, although the investigator named is recognized as a competent physician, he claimed several years ago (again without formal publication) to be able to control the sex of human offspring by a technique which has been largely discredited. For these reasons, the scientific reliability of the above report is in serious question.

Studies of *In Vitro* Fertilization in Nonhuman Primates

When the present studies were initiated, there were only two reported attempts of *in vitro* fertilization in nonhuman primates. *Saimiri sciureus* was the species used in both cases. Johnson, Harrison and Dukelow (1972a) first reported, in an abstract, sperm attachment to

the zona pellucida of *S. sciureus* oocytes and later a more detailed account was published by Johnson (1972b). These investigators cultured *S. sciureus* follicular oocytes in TC 199 plus 25 µg/ml estrone sulfate with either 20% heat inactivated squirrel monkey serum or agamma fetal calf serum using an *in vitro* fertilization system similar to that employed with rabbits. Of four oocytes cultured with spermatozoa, one showed sperm attachment in the zona pellucida and two had sperm in the cumulus cell masses or attached to them.

In the other study, Cline, Gould and Foley (1972) reported, in an abstract, fertilization *in vitro* of mature *S. sciureus* ooctyes. They used a medium of TC 199 with 20% heated agamma newborn calf serum plus bovine serum albumin. Of 16 mature oocytes recovered from pregnant mare's serum (PMS) treated squirrel monkeys, 7 appeared fertile, 5 of which developed to the 2-cell stage. In an expanded report, 22 mature ova were utilized in an *in vitro* fertilization system employing preincubation of ejaculated spermatozoa in follicular contents plus medium prior to insemination into test cultures (Gould, Cline and Williams, 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 2-cell stage. Twenty-four oocytes were reported to have matured *in vitro* to metaphase II but showed no signs of fertilization. The authors stated that the success or failure of individual trials was closely associated to the duration of sperm survival in the medium, but they provided no confirming data.

After the present studies were undertaken, attempts have been made at developing an *in vitro* fertilization system for another species of nonhuman primate. *Papio cynocephalus* follicular oocytes have been 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 50% Brinster's medium (Kraemer, 1973). 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 to *in vitro* fertilize these oocytes was made. Instead, follicular oocytes recovered from the ovaries of gonadotropin-primed baboons were cultured in a variety of fertilizing media including follicular fluid, Brinster's medium or Bavister's medium. All received either uterine incubated spermatozoa or Bavister's medium with fresh or cervical mucus-incubated spermatozoa. Incubations were for 8 hours followed by incubation in 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 with spermatozoa from Brinster's and Bavister's media and were found to have 2 polar bodies and 2 pronuclei. One 6-cell ovum was recovered after fertilization in Bavister's medium with fresh and cervical mucus incubated spermatozoa. Nuclei were visible in all 6 cells. These results are preliminary and await duplication.

Attempts at *in vitro* fertilization and embryo transfer have not been reported for nonhuman primates. However, Suzuki and Mastroianni have matured *Macaca mulatta* oocytes *in vitro* in Waymouth's medium plus

10% Rhesus serum and then transferred them to oviducts of inseminated females. Of 31 oocytes matured, transferred, and recovered 24 to 48 hours later, 15 were definitely not fertilized, 8 were degenerated, 6 contained 2 polar bodies or 2 pronuclei and 2 contained two degenerating blastomeres each. The authors suggested that improper timing of transfer following *in vitro* culture, failure of cytoplasmic maturation, and less than ideal intraluminal oviductal conditions were factors which may have contributed to the lack of fertilization. The same factors may be important to future work on developing embryo transfer techniques.

Materials and Methods

Animals

S. sciureus of the Brazilian type (export point of origin: Leticia, Columbia) were purchased from Tarpon Zoo, Inc., Tarpon Springs, Florida. Seventeen females, in captivity from 17 to 24 months at the start of the study, were housed either in stainless steel, flush type cages during the period of time they were being used in an *in vitro* fertilization trial (usually about two weeks) or in a large gang cage (Harrison, 1973). Both types of caging have already been described in this thesis (page 14). Three males were employed in these experiments and housed in the stainless steel cages. One of the males was acquired after the start of the study and therefore used only in the later trials. The animals were fed commercial monkey feed (Wayne Monkey Diet, Allied Mills) and received fresh water *ad libidum*. The animal room had fluorescent lighting on a 12:12 light-dark cycle and was temperature controlled at 21°C ± 2°. Relative humidity was not controlled.

Ovulation Induction Procedure

Mature female squirrel monkeys were induced to ovulate without regard to their natural cycle using a regime previously reported by Dukelow (1970). This regime was modified for the present study to include only the four daily intramuscular injections of FSH (1 mg, FSH-P, Armour-Baldwin Laboratories, Omaha, Nebraska) with an intramuscular injection of 500 i.u. HCG (A.P.L., Ayerst Laboratories, New York, New York) on the early morning of the fifth day. No progesterone pretreatment was given.

Recovery of Sperm

Male squirrel monkeys were electroejaculated using the method and restraint system described by Kuehl and Dukelow (1973). This procedure is discussed in detail in the paper which has been included as the first part of this thesis (page 4). The ejaculate, in the form of a coagulum, was incubated in 1 to 2 ml of culture medium (see page 40) at room temperature for 0.5 to 2 hours. The resulting sperm suspension was aspirated from the sample tube containing the coagulum and used to inseminate oocyte cultures. A small sample of sperm suspension was counted using a heomocytometer slide to ascertain the concentration of sperm. The suspension was also observed to determine percentage of motile sperm and the percentage of normal-appearing sperm at the time of insemination. Any type of abnormal sperm was noted. After the quality of the sperm suspension was determined, the oocyte cultures were inseminated.

Recovery of Oocytes

Laparotomies were performed four to twelve hours after HCG administration. The ovaries were exposed by midline abdominal incision, while the monkeys were under sodium pentobarbital anesthesia (16.2 mg/animal of Halatal, Jensen-Salsbery Laboratory, Kansas City, Missouri). The ovaries were examined for ovulation points and follicular development. All visible follicles were aspirated using a 1 ml tuberculin syringe containing 0.1 ml of culture medium. Follicles were punctured laterally with a 25 gauge needle (bevel side down) and the contents aspirated into the syringe. Following follicle aspiration the incision was closed by suturing first the muscle layers then the skin with a chronic 3-0 suture. Nitrofurazone powder was placed on the incision and then covered with nitrofurazone ointment. Each monkey also received 1.5×10^4 units of procaine penicillin G intramuscularly and was kept warm with a heating pad until completely recovered from the anesthetic.

The oocytes recovered by aspiration along with the associated cumulus mass, follicular fluid and medium (see page 40) were placed in one of the two types of culture containers. The type used most often was a 30 X 13 mm sealable culture dish (No. 4340, A. H. Thomas Company) in which the aspirated fluid was placed along with the sperm suspension and covered with light viscosity silicone fluid (Dow Corning 200 fluid, Dow Corning Co., Midland, Michigan) previously sterilized and equilibrated with 5% CO_2 in air. The culture dishes were incubated at 37°C in a moist atmosphere of 5% CO_2 in air and checked at intervals with an inverted microscope for evidence of fertilization. Sperm motility and

signs of bacterial contamination were also observed. The other type of culture container was a chamber-slide (tissue culture chamber/slide, No. 4808, Lab-Tek Products, Naperville, Illinois) with eight chambers. The ova and sperm media were placed in the chambers, but were not covered with silicone fluid. The slides were incubated in the same manner as were the dishes, but provided for better visual observations while using the inverted microscope.

At the end of the incubation period the ova were recovered from the culture containers using Pasteur pipettes while being observed with a disecting microscope. The ova were then observed both live and after staining by lacmoid (method of Iwamatsu and Chang, 1972). Photographs of oocytes were taken during and after the culture period. All equipment other than the sterile plastic syringes and sterile culture slides were autoclaved. Gamete handling was done under a hood (tissue culture hood, Lab Con Company) equipped with an ultraviolet light for sterilizing the working surfaces and a heating apparatus for maintaining temperature at about 37°C.

The Culture Medium

A variety of culture media were used in these studies. They were as follows:

1. Brackett's medium for rabbit *in vitro* fertilization supplemented with 20% heat inactivated female squirrel monkey serum,
2. TC 199 (Grand Island Biological Co., Grand Island, New York) supplemented with 20% heat inactivated agamma calf serum (North American Biologicals, North Miami, Florida),

3. A combination of media 1 and 2 above at a 1:1 level,
4. Brinster's medium unsupplemented, or
5. Ham's F 10 (Grand Island Biological Co.) supplemented with either 10% or 20% heat inactivated agamma calf serum.

All media contained 50 units of penicillin-G/ml. The media were sterilized by use of millipore filters (H.A. 0.45 μ, 25 mm diameter, Millipore Corp.).

Criteria for Fertilization

The determination of fertilization was made when any one of the following conditions was met:

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

Criteria two through four had to be verified by several observations of the oocytes in culture so that fragmented ova were not mislabeled as multicelled embryos. When a culture vessel contained more than one oocyte, the relative positions of each of the oocytes to the sides of the vessel were noted. Thus an individual oocyte could be identified at each observation.

Experimental Design

The individual experiments undertaken in this research were conducted such that the conditions of one experiment were based on the results of the previous one. Thus a search strategy was evolved to find a system that would support *in vitro* fertilization. In essence, then, each experiment had the previous one as a control in addition to controls within each experiment. For example, when sperm motility was observed in oocyte cultures, sperm motility in cultures without oocytes and follicular contents were also noted. When changes in the oocytes that were cultured with sperm were observed, oocytes cultured without sperm served as a control. By changing only one known variable from one experiment to the next, important variables could be isolated. However, many variables were difficult to control, such as gonadotropic response of the female ovaries or spermatozoa quality. Also important variables can easily change if they were unknown or not measured. For this reason as much data were collected for each experiment as possible including those based on subjective judgments. As experiments progressed new measures were added to the data set collected.

A further problem existed when comparing quantal data between sets of experiments. Oocytes either mature or do not mature and are either fertilized or not fertilized at the end of the culture period. The quantal nature of the response and the differences in ovum numbers between studies do not allow statistical analysis by analysis of variance. Care must be taken to ensure that a continuum of data points is possible for use of many conventional tests such as the student's t or

the chi-square tests. Non-parametric methods may be used in some cases but because of the lack of general acceptance, these tests were avoided. If adequate ovum numbers were used in each treatment, statistical analysis allowed for testing of variables.

Results

One hundred and seventy-six follicular oocytes were recovered by aspiration from 719 follicles on the ovaries of seventeen animals. Table 6 shows an accounting of individual animals in terms of the number of times used and number of oocytes recovered. The mean number of oocytes recovered per animal was 3.9 which is 24% of the mean number of follicles aspirated, 16.0.

S. sciureus follicular oocytes were cultured in a variety of media. Table 7 shows the results of *in vitro* fertilization experiments conducted in the different media. This table provides comparison data in terms of the proportion of oocytes that were atretic, the proportion of nonatretic oocytes that matured to the first polar body stage and were available for fertilization, and the proportion of the matured oocytes that were fertilized. Atretic oocytes were defined for the comparisons as those oocytes showing darkening, vesicularization and shrinkage of the ooplasm or uneven division of the ooplasm (fragmentation) at the time of the first observation of the culture. Other variables of the *in vitro* fertilization culture system were also evaluated using the same kind of comparisons. Among these variables are the length of culture, the type of sperm present during culture, and the source of the oocytes that were cultured.

Table 6. Recovery efficiency of oocytes from gonadotropin treated female squirrel monkeys

Animal number	Times used	Total oocytes recovered	Total follicles aspirated	Percent recovery
1301	1	4	8	50
1308	1	0	13	0
1317	2	4	23	17
1323	1	13	30	43
1332	1	3	16	19
1333	4	23	61	38
1334	4	7	60	12
1335	5	18	81	22
1336	5	19	66	29
1337	5	17	106	16
1339	4	22	84	26
1342	1	2	12	17
1344	3	14	47	30
1347	1	1	7	14
1348	2	11	48	23
1349	3	13	45	29
1397	2	5	12	42
Totals	45	176	719	
Means per surgery		3.9	16	24

Table 7. Effect of medium on *in vitro* fertilization cultures

Medium	Total ova	Total cultures	Atretic ova	Percent of Total	Matured ova	Percent of non-atretic	Fertile ova	Percent of matured
TC 199 + 20% agamma calf serum	83	40	29	35	27	50	9	33
F 10 + 20% agamma calf serum	11	5	2	18	7	78	2	29
Brackett's + 20% S. monkey serum	17	6	6	35	4	36	1	25
Brackett's and TC 199 + 20% a. calf serum	38	12	1	3	10	27	2	20
Brinster's medium	3	1	1	33	0	0	0	0

Table 8 shows the data for the four periods of culture (24 hours, 48 hours, 52 to 57 hours and 73 to 78 hours). The sperm conditions present during the culture period were: (1) no sperm, (2) motile sperm (for 24 hours or more) that had no prior treatment before insemination into the cultures, (3) motile sperm (for at least 24 hours) that had been incubated 2 hours with oocytes, cumulus cells, and follicular fluid prior to insemination into the cultures, and (4) motile sperm for less than 24 hours. The results of the various sperm treatments are shown in table 9. All sperm used for insemination of the 60 cultures receiving sperm in table 9 were from two males of proven fertility. Immature sperm, those with a cytoplasmic droplet still present on the midpiece, were found in all ejaculates and constituted 50 to 90% of all sperm inseminated into the cultures of oocytes. Fertile ova were found in cultures inseminated with 90% immature sperm, so high proportions of immature sperm were not taken to indicate low quality ejaculates. Oocytes used for culture were aspirated along with associated follicle cells and follicular fluid. There were two parameters for follicle evaluation. The first was size based on the volume of follicular fluid aspirated. The effects of follicle size on the fate of oocytes in culture is shown in table 10. The second parameter was the presence or absence of blood cells in the aspirate from the follicle. Follicles hemorrhagic in appearance had a high amount of blood in the follicular contents and thus in the culture. Amounts of blood were considered high when the culture medium took on a red color. The effects of this are shown in table 11.

Table 8. Effect of length of culture on the fate of ova in *in vitro* fertilization cultures

Length of culture	Total ova	Total cultures	Atretic ova	Percent of Total	Matured ova	Percent of non-atretic	Fertile ova	Percent of matured
24 hours	75	32	18	24	23	34	6	26
48 hours	24	10	3	13	8	38	2	25
52 to 57 hours	29	13	10	35	5	26	1	20
73 to 78 hours	24	9	8	33	10	63	5	50

Table 9. Effect of sperm conditions on the fate of ova in *in vitro* fertilization cultures

Sperm conditions	Total ova	Total culture	Atretic ova	Percent of Total	Matured ova	Percent of non-atretic	Fertile ova	Percent of matured
No sperm	7	3	3	43	3	75	0	0
Non-pretreated sperm remaining motile at least 24 hours	74	31	21	28	20	38	9	45
Pretreated sperm remaining motile at least 24 hours	13	3	4	31	4	44	2	50
Sperm not remaining motile at least 24 hours	53	26	10	19	19	44	3	16

Table 10. Fate of ova from follicles of varying sizes in *in vitro* fertilization culture

Source of ova	Total ova	Total cultures	Atretic ova	Percent of Total	Matured ova	Percent of non-atretic	Fertile ova	Percent of matured
Unclassified follicles	83	34	16	19	28	42	6	21
Large follicles[a]	18	9	9	50	6	67	2	33
Medium follicles[b]	17	10	7	41	3	30	0	0
Small follicles[c]	28	12	4	14	10	42	6	60

[a]Contain in excess of 0.02 ml follicular fluid.

[b]Contain between 0.02 and 0.01 ml of follicular fluid.

[c]Contain less than 0.01 ml follicular fluid.

Table 11. Fate of ova from blood follicles in *in vitro* fertilization culture

Source of ova	Total ova	Total culture	Atretic ova	Percent of Total	Matured ova	Percent of non-atretic	Fertile ova	Percent of matured
Blood follicles	46	23	12	26	15	44	7	46
Nonbloody follicles	102	42	26	26	31	41	7	23

The type of culture chamber was changed near the end of the study from a relatively large volume (1 to 3 ml) culture dish to a small volume chamber-slide. This change reflected the results of experience gained in developing an *in vitro* fertilization system. Table 12 shows a comparison of the chamber-slide and the culture dish methods.

Photographs were taken both during and after the oocyte culture. Some oocytes were fixed and stained with lacmoid. These ova were also photographed. Figures 9 through 17 show a progression of oocytes from a single cell stage to a four-celled embryo. In figures 10 through 13 lacmoid stained oocytes are used to depict maturation from a single-celled oocyte arrested at the germinal vesicle stage to a single polar body or metaphase II oocyte ready for fertilization.

Figure 18 shows a summary of the observations of *in vitro* fertilization of *S. sciureus* follicular oocytes. Points of development are plotted as a function of the time they were first observed in the *in vitro* fertilization culture. These points reflect both the types of ova development that occur through time and the intervals of observation of the oocytes in culture. The maturation to metaphase II is plotted through time following HCG administration. All other stages of development are plotted in time following *in vitro* insemination. Data for human oocyte development in culture following *in vitro* insemination (Edwards, 1973) is provided for comparison.

Table 12. Effect of culture container and associated changes in handling technique on the fate of ova in *in vitro* fertilization culture

Type of culture	Total ova	Total cultures	Atretic ova	Percent of Total	Matured ova	Percent of non-atretic	Fertile ova	Percent of matured
Chamber/slide	19	14	3	16	9	56	6	67
Culture dish	152	64	39	26	48	42.5	14	29

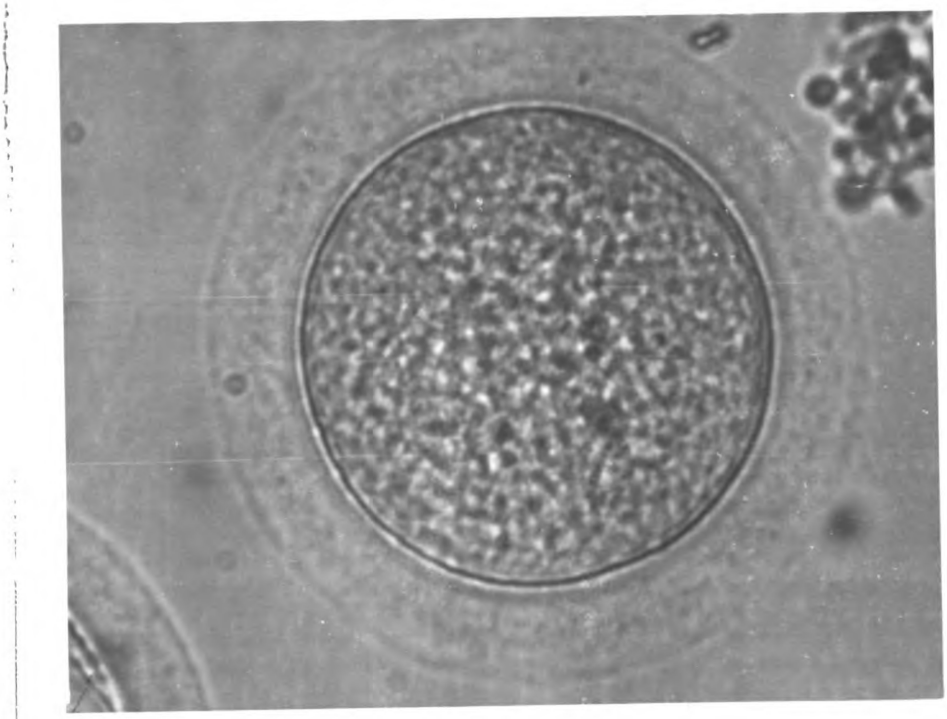


Fig. 9. One-celled follicular oocytes from *Saimiri sciureus* (X 300).

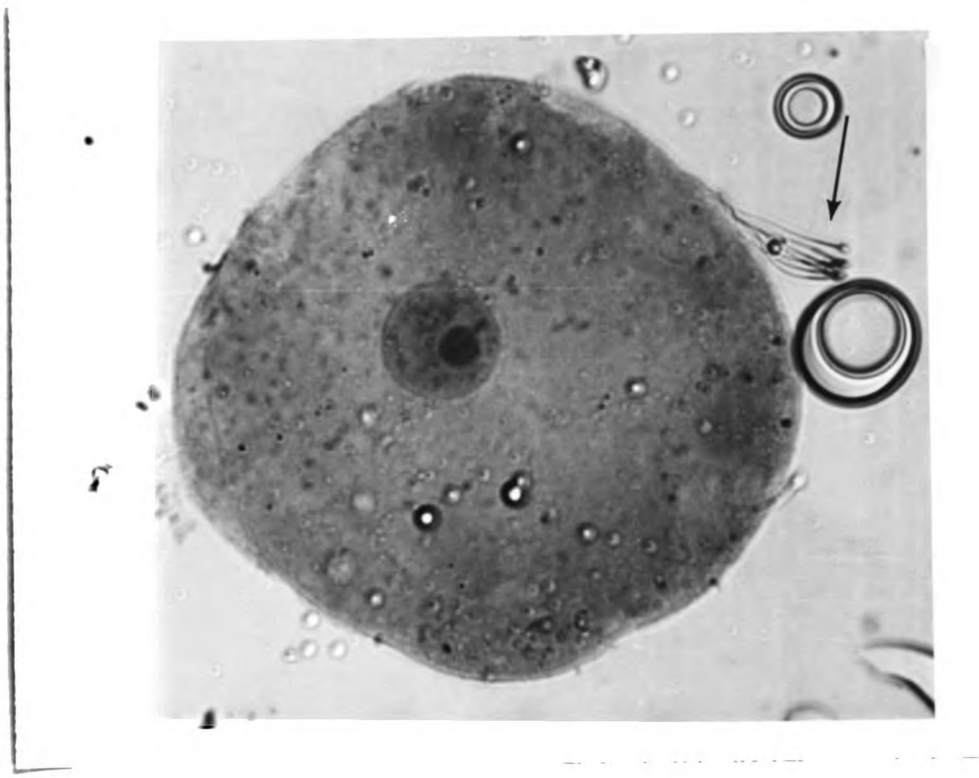


Fig. 10. One-celled oocyte stained with lacmoid to show a centrally located germinal vesicle. Arrow indicates stained spermatozoa also in view (X 300).

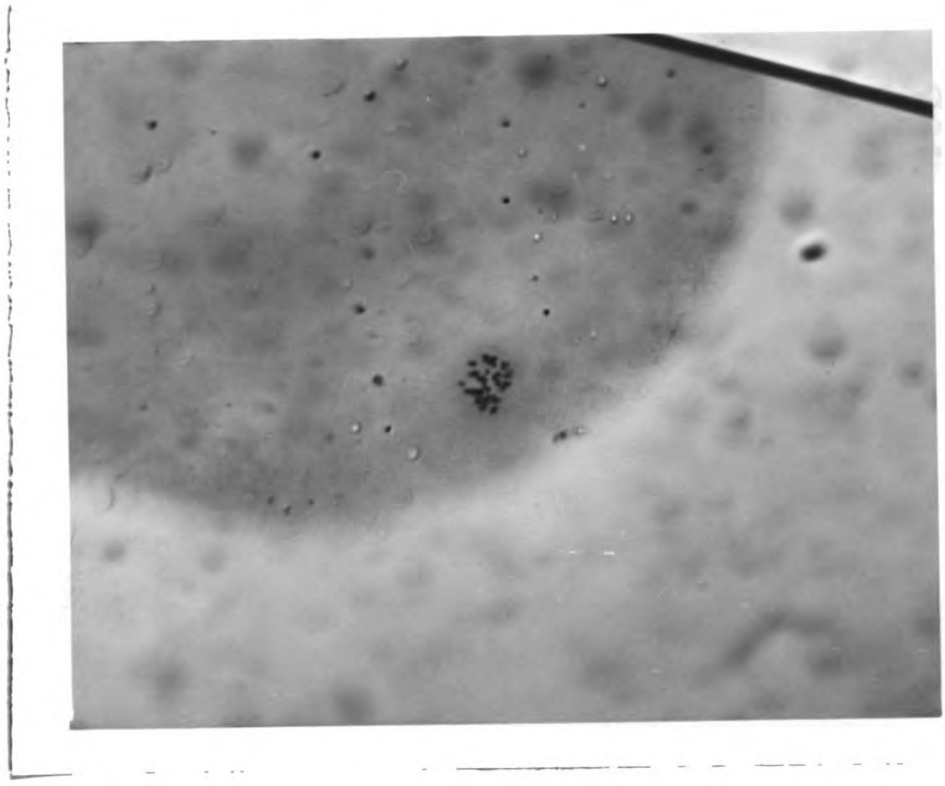


Fig. 11. Maturing *S. sciureus* oocyte showing a metaphase I chromosome spread (lacmoid stained) (X 375).

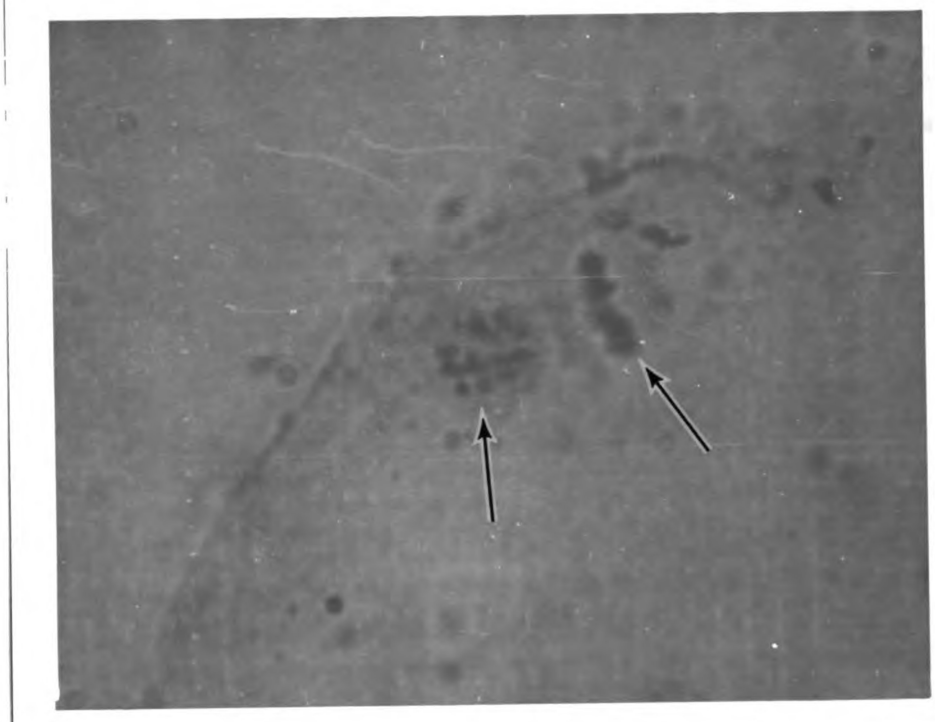


Fig. 12. Oocyte in late anaphase I stage prior to emitting a first polar body. Arrows indicate the two separate groups of lacmoid stained chromatin material (X 800).

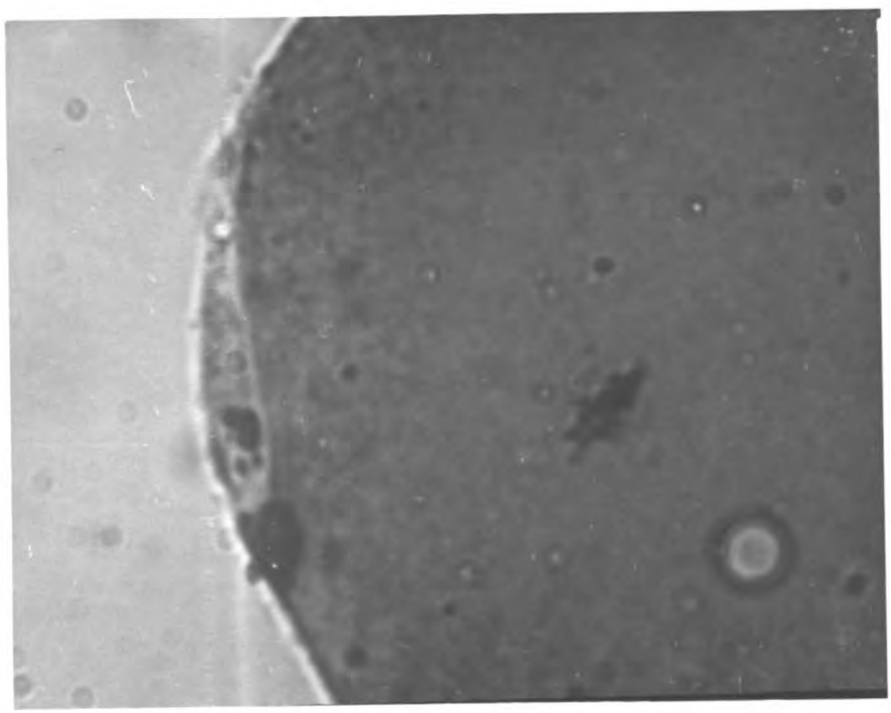


Fig. 13. Lacmoid stained *S. sciureus* follicular oocyte showing the chromatin of the first polar body and a metaphase II grouping in the ooplasm (X 800).

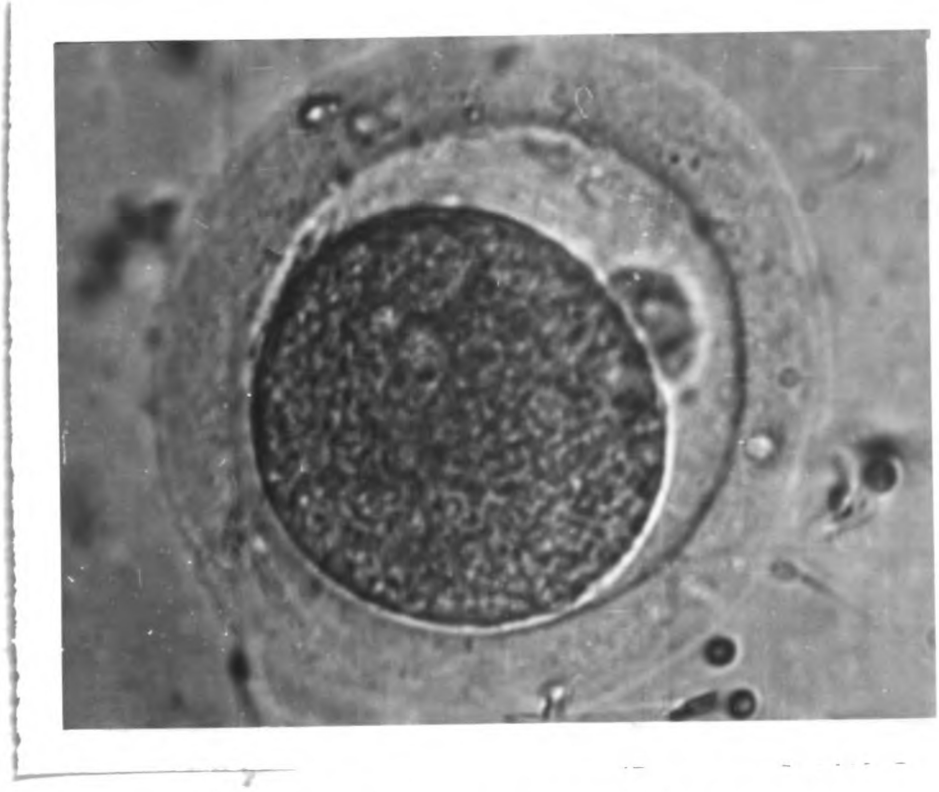


Fig. 14. *S. sciureus* oocyte in the first polar body stage with sperm sticking to the zona pellucida (X 300).

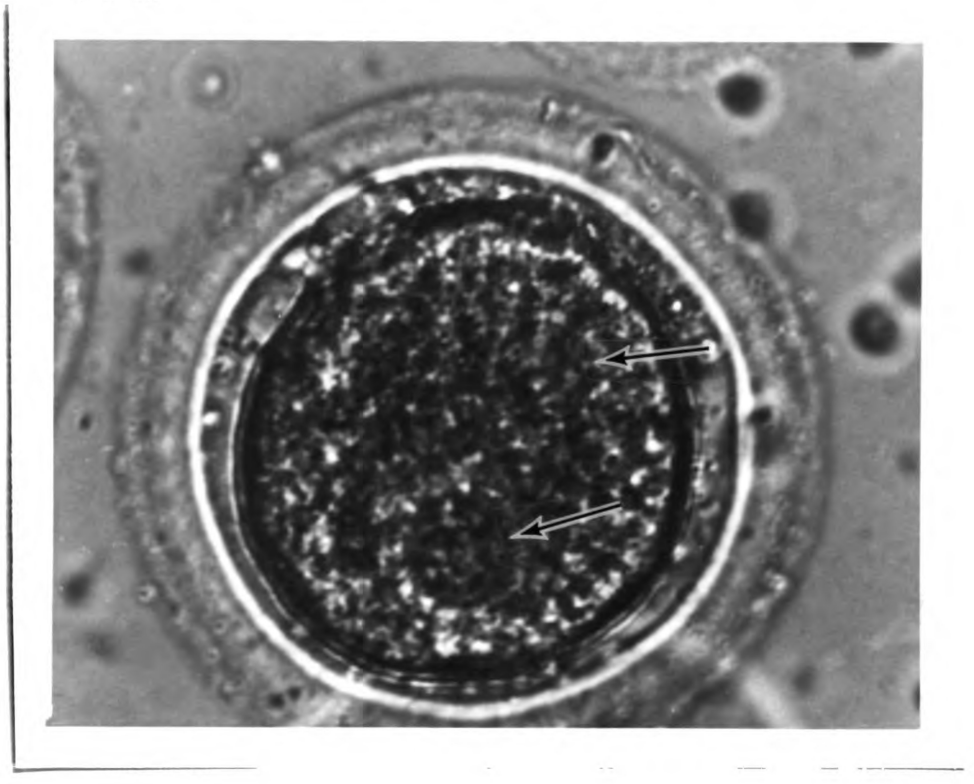


Fig. 15. Oocyte with two polar bodies and two pronuclei (indicated with arrows) (X 300).

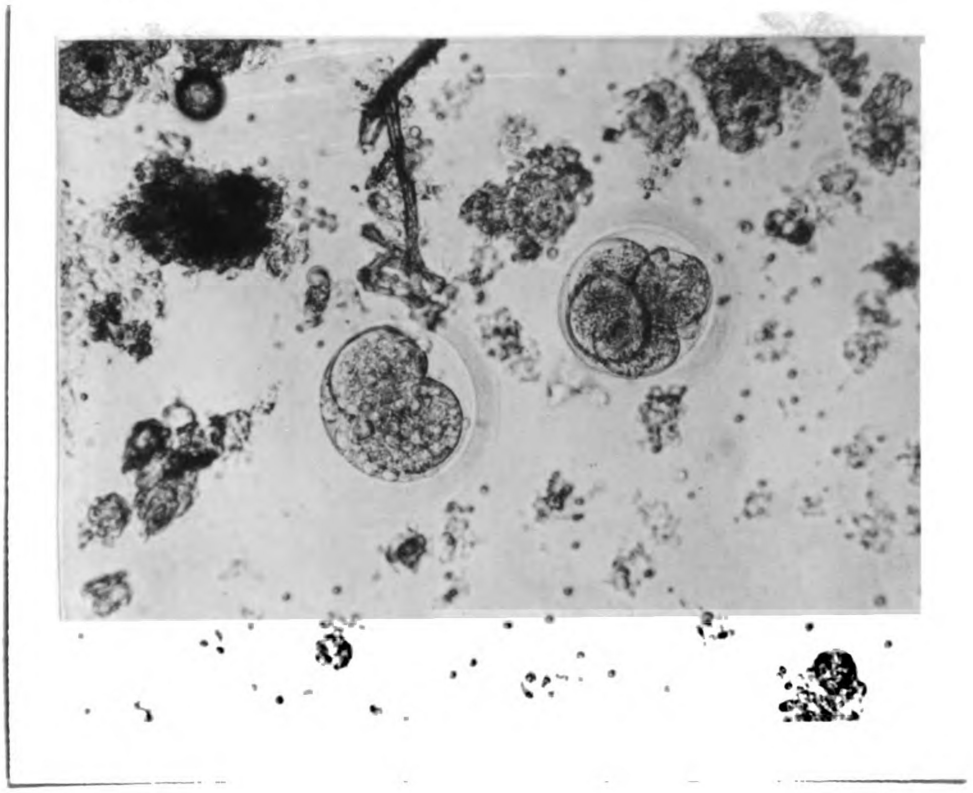


Fig. 16. Two- and four-cell cleaved *S. sciureus* oocytes 48 hours after insemination and culture *in vitro* (X 100).

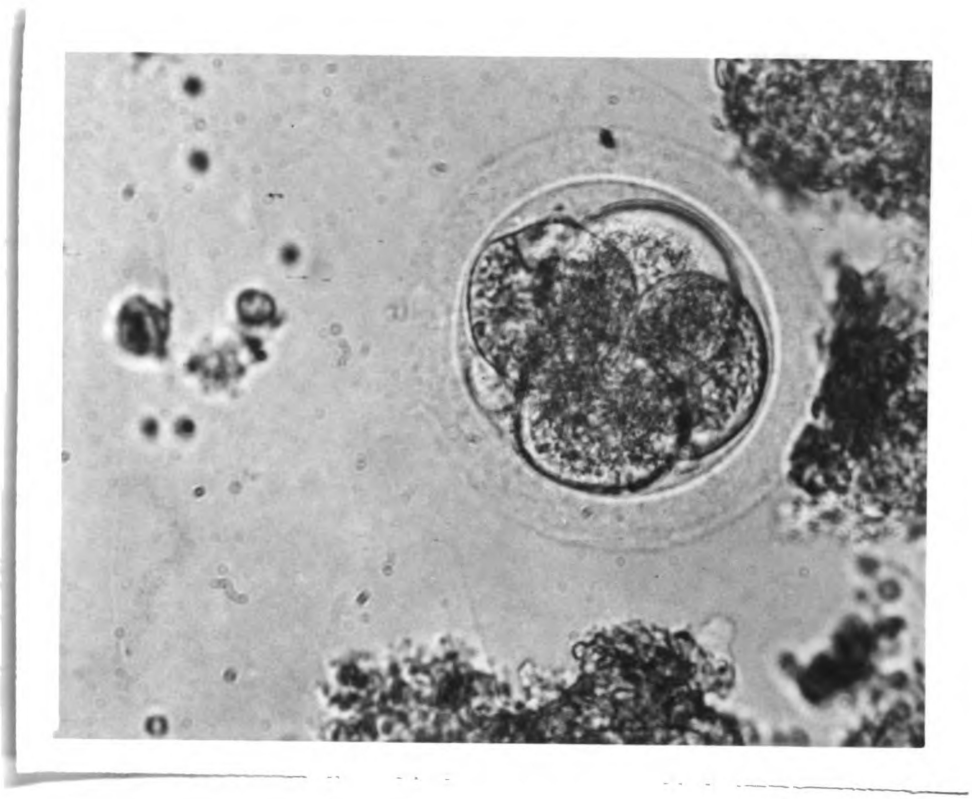


Fig. 17. Four-celled cleaved *S. sciureus* oocyte 48 hours after insemination (X 200).

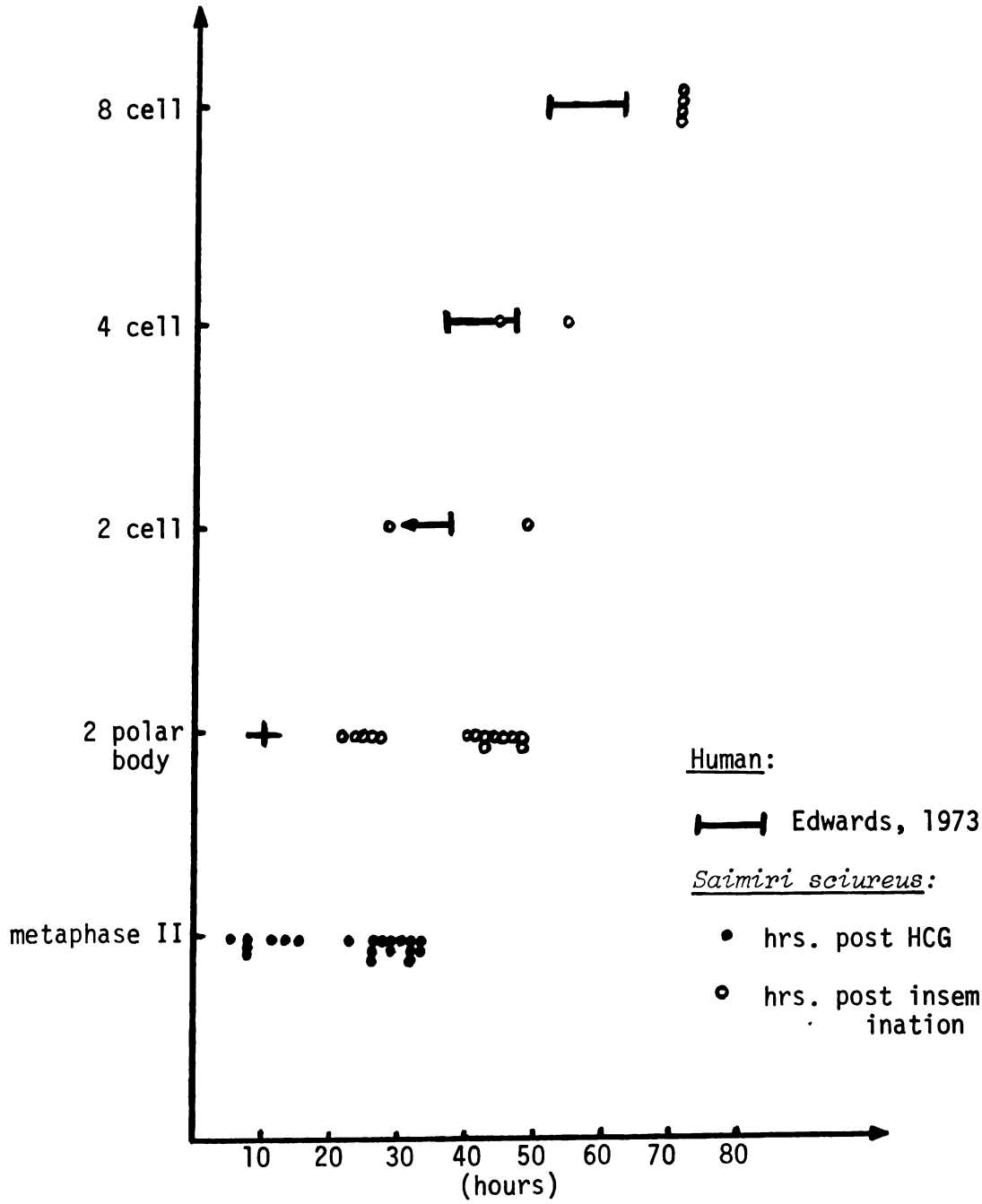


Fig. 18. Timing of *Saimiri sciureus* fertilization and cleavage *in vitro*.

Discussion

The results of the search strategy to develop an *in vitro* fertilization system have been described in the preceding section (page 43). *In vitro* fertilization of *S. sciureus* follicular oocytes was accomplished. Development of the fertilized embryos through the four-cell stage was observed in several different systems. The *in vitro* fertilization system can be broken down into a set of four procedures: (1) recovery of ova, (2) recovery of sperm, (3) combining the gametes in culture to obtain fertilization, and (4) transfer or maintenance of the fertilized embryos. The importance of various parameters to the system will be discussed as they relate to the total process of *in vitro* fertilization.

Female squirrel monkeys were treated with a standard ovulation induction regime to provide a supply of follicles to be aspirated. Animals can be used several times. For instance, in the present study animals have been used as many as five times in a year. An ovary produced, on the average, 8 follicles of different sizes. In the present study 24% of these follicles yielded oocytes, but near the end of the study 30 to 50% recoveries of oocytes were not uncommon. This shows that experience in the aspiration procedure can increase the number of oocytes recovered and thus those available for culture.

The stage of follicular development appears to be important to the oocyte development *in vitro*. Table 10 shows that as follicle size increases the number of atretic oocytes from the follicles increased such that 50% of the oocytes from large follicles were atretic. However,

the proportion of nonatretic oocytes that matured was highest for large follicles. This is consistent with the theory that large follicles are either close to ovulating (thus their oocytes are mature) or in the process of degenerating. The small follicles seem not to have been stimulated to initiate complete development. Fewer small follicles had atretic oocytes and more small follicles had oocytes that retained the ability to mature when comparing the same parameters to the larger, perhaps older, medium sized follicles. Furthermore, while many ova from the large follicles were aspirated in the one polar body stage, few ova from small follicles were mature. The high proportion of oocytes from small follicles that matured *in vitro* and were fertilized is in direct opposition to the observation of Gould, Cline and Williams (1973) which indicated that oocytes which matured *in vitro* were not fertilizable. The fate of oocytes from blood follicles (table 11) was no different than that of ova from normal follicles. The exception was in the ability of oocytes to be fertilized. As both kinds of ova mature at the same rate, it was thought that some blood components aided in the cultures to increase fertilization.

The recovery of male gametes was aided by the use of a restraint device. Because two and sometimes three males were used to provide sperm, there was no problem with supply. No means of assessing ejaculated sperm quality prior to use for *in vitro* fertilization was found. Once in culture, maintenance of motility was most important (table 9). If sperm motility ceased prior to the end of the first day of oocyte culture, the incidence of fertilization decreased. This may be due to

a lack of motile, fertile sperm at a time when oocytes are maturing in culture. There was little difference in fertility between pretreated sperm and nonpretreated sperm *in vitro*. This does not rule out the possibility of capacitation occurring. Gwatkin, Anderson and Hutchinson (1972) have shown that cumulus cells of the hamster and bovine are capable of capacitating hamster sperm *in vitro* as evidenced by their ability to fertilize cumulus-free ova *in vitro*. A similar event has recently been proposed to occur during *in vitro* fertilization of human follicular oocytes (Soupart and Strong, 1974). Some component of the follicular contents appeared to be important in maintaining *S. sciureus* sperm motility in culture as evidenced by "sperm only" controls. In all cases where sperm were cultured *in vitro* in medium alone, the loss of motility was quicker for the sperm cultured in medium alone than for the sperm cultured with the follicular contents.

The culture period, where sperm and oocyte units, was examined with regard to length of culture and medium of culture. From table 8 the conclusion may be drawn that the longer the period of culture the greater the proportion of oocytes maturing and becoming fertile. The highest fertility was reached after more than 72 hours of culture. The culture medium was the only variable factor in the cultures environment as temperature and atmosphere were maintained at 37°C and 5% carbon dioxide in moist air. A number of different culture media were tried (table 7), but TC 199 or F 10 supported the highest proportions of maturation and fertilization. Both media were supplemented with 20% heat-inactivated agamma newborn calf serum and were, thus, undefined.

Follicular contents were also present in the culture media. Although the pH of the culture media at the beginning of culture was between 7.2 and 7.4, it is possible that changes during the culture period could have influenced fertilization or maturation. Osmolarity of culture media was not monitored. As an unknown parameter osmolarity could be playing an important part in the culture. It was noted many times that oocytes removed from culture medium and placed in saline would show ooplasmic shrinkage. Sperm, on the other hand, maintained their motility in saline as long as an energy source was provided.

An alternate culture procedure was used employing a chamber-slide. F 10 medium supplemented with 10% heat-inactivated agamma calf serum was used as a medium. With this medium sperm motility was maintained in excess of 48 hours. The chamber slide had the advantage of improved visibility of oocytes during culture so that their development could be followed. As shown in table 12 both maturation and fertilization increased to levels higher than those with the previously used technique.

Maintenance of development of the fertilized embryo from a 2 pronucleate, 2 polar body stage through the four-cell stage was obtained using either TC 199 or F 10 based media. Both media have been reported to give good results for both human and squirrel monkey oocytes. Figure 18 shows a comparison of the observations made on developing *S. sciureus* embryos with those for human embryos. There appears to be a good correlation between the data. The *S. sciureus* observations were generally later in time; however, this may be accounted for by differences in the intervals of observation of the two types of data.

S. sciureus oocytes do appear to mature faster than either human or rhesus monkey oocytes which are reported to require more than 30 hours to mature to metaphase II (Edwards, 1973).

The chamber-slide and associated techniques provide a system for *in vitro* fertilization of *S. sciureus* oocytes. This system may prove useful in future studies involving transferring fertilized embryos as it allows for good observation of the embryos as they develop. Thus, embryos at specific stages of development could be gathered and subsequently transferred to recipient animals. The eight chambers on a slide allow culture conditions to be varied and comparison studies to be made. Finally the culture system offer a previously unavailable opportunity to study fertilization in a nonhuman primate using a variety of experimental manipulations which can still be properly controlled.

SUMMARY AND CONCLUSIONS

Saimiri sciureus was used as a model nonhuman primate for development of an *in vitro* fertilization system. Associated problems of male restraint during electroejaculation and female low ovulatory response period were also studied. The following conclusions are indicated by the data obtained:

1. A simple restraint device can be constructed and used by a single operator to collect semen samples from unanesthetized squirrel monkeys.
2. Increased duration or dose of FSH in the standard ovulation induction regime will increase the ovulatory response of squirrel monkeys during the season of low response, chiefly by increasing the proportion of animals ovulating.
3. A system has been developed for fertilizing squirrel monkey follicular oocyte *in vitro* and supporting embryonic development to the four-cell stage.

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Full Papers

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

Abstracts

- See the following pages for texts of abstracts.

Fed. Proc. 32:283A, 1973

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

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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 effect 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 sciureus)[1]

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A restraint device designed to allow a technician 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.

(Supported by NIH Career Development Award No. 1-KY-HD35, 306-01.)

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[1]Presented at the 24th Annual Mtg. Amer. Assoc. for Lab. Anim. Sci., Miami Beach, Florida, October 1-5, 1973.

Fed. Proc. 33, 281A, 1974

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

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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 IT'S RELATIONSHIP TO BIRTH DEFECT RESEARCH[1]

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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]To be presented in the Proceedings at the 5th International Congress of Primatology, Nagoya, Japan, August, 1974.

For 38 animals, 273 follicular oocytes were recovered by aspirating 654 follicules 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.

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