THESIS

This is to certify that the

thesis entitled
Culture Technique and Cyclic Nucleotide Effects on in vitro fertilization of the squirrel monkey (Saimiri sciureus)

presented by

Philip J. Chan Chee Sem

has been accepted towards fulfillment of the requirements for

M.S. degree in Physiology

W. Richard Dukelow

Major professor

Date May 8, 1981

CULTURE TECHNIQUE AND CYCLIC NUCLEOTIDE EFFECTS ON IN VITRO FERTILIZATION OF THE SQUIRREL MONKEY (*SAIMIRI SCIUREUS*)

By

Philip J. Chan Chee Sem

A DISSERTATION

Submitted to

Michigan State University

In partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Physiology

1981

ABSTRACT

CULTURE TECHNIQUES AND CYCLIC NUCLEOTIDE EFFECTS

ON IN VITRO FERTILIZATION OF THE SQUIRREL MONKEY (SAIMIRI SCIUREUS)

By

Philip J. Chan Chee Sem

The use of non-human primates as models for in vitro fertilization and development is important for studying the events involved in primate reproductive physiology. A squirrel monkey in vitro fertilization system has been developed successfully with a laparoscopic oocyte recovery rate of 41.0% and mean maturation, fertilization and cleavage rates of 70.3, 54.8 and 28.6% respectively. Some embryos developed to the 16-cell stage. Maturation was highest from March to June. Corona radiata cells promoted oocyte maturation. Follicular fluid increased the fertilization rate. Sperm studies indicated that DL-norepinephrine, caffeine and dibutyryl cyclic AMP (dbcAMP) stimulated motility. Imidazole and dbcGMP inhibited motility. The involvement of cAMP in the activation of the squirrel monkey sperm was postulated. The addition of dbcAMP to in vitro cultures increased fertilization rates from 60 to 90 percent but did not affect cleavage rates. Dibutyryl cyclic GMP (dbcGMP) decreased fertilization rates. In a hamster in vitro fertilization system dbcGMP increased fertilization rates while dbcAMP had no effect. The difference noted may be a species-difference phenomenon.

To my parents...

To my Chief...

To Dr. Soupart...

and to all in vitro scientists.

ACKNOWLEDGMENTS

I wish to express my deepest gratitude and extend my very special thanks to Dr. W. Richard Dukelow for his encouragement, assistance and patience, that I may learn to be a skilled scientist.

Thanks go to Bonnie Cleeves for her help and sunshine at the Endocrine Research Unit. I also wish to thank Dr. Hisatomi Mizoguchi and Dr. Takehito Asakawa for their scientific help, and everyone else at the Endocrine Research Unit for their friendship.

I wish to thank the members of my committee, Dr. H. A. Tucker, Dr. E. Rivera and the late Dr. W. D. Collings for their assistance and advice.

My warmest appreciation goes to my parents, Mr. and Mrs. Chan Hon Yew, my sister and my brother for their love and understanding through the years.

Finally, I wish to give a standing ovation of thanks to the greatest love of my life.....

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

Introduction

The use of an in vitro system for studies on fertilization and development has been accomplished in the laboratory species, several domestic animals and in primates including humans. In vitro cultured embryos have been successfully transferred to recipient mothers with subsequent live births with several species.

The achievement of fertilization outside the reproductive tract enables one to observe and monitor the development of the embryos. In addition, the metabolic activity, the biochemical changes and the chromosomal normality can be studied in an in vitro culture system. Clinically the in vitro culture system could be used for overcoming infertility problems such as salpingitis or low sperm concentrations. It could further be used for assessing sperm fertility or effects of pharmacological drugs.

Golden hamsters and squirrel monkeys were used in the present studies. The less expensive hamster was used to provide oocytes for preliminary screening trials. The squirrel monkey being a nonhuman primate, provided a model for the in vitro fertilization studies that have greatest relevance in terms of revealing the complexities of the reproductive events in man.

The objectives of the in vitro fertilization studies were:

1) To confirm and improve the techniques for in vitro fertilization.
2) To quantify such variables as seasonality, recovery and insemination times, efficiency of oocyte recovery from left and right ovaries and the effect of follicular fluid and cumulus cells on in vitro fertilization.

3) To determine the effects of epinephrine, norepinephrine, sperm motility factor (SMF), caffeine, dibutyryl cyclic AMP (dbcAMP), dibutyryl cyclic GMP (dbcGMP) and imidazole on squirrel monkey sperm motility.

4) To examine the effects of circulating medium, continuous medium flow, dbcAMP and dbcGMP on a hamster in vitro fertilization system.

5) To investigate the effects of medium replacement, Gentamicin®, dbcAMP and dbcGMP on the squirrel monkey in vitro fertilization system.

Culture techniques and cyclic nucleotide effects on in vitro fertilization of the squirrel monkey (Saimiri sciureus).

LITERATURE REVIEW

I. Stimulation of sperm motility and acrosome reaction by methyl xanthines.

The presence of low molecular weight (100-200) heat-stable factors that stimulate sperm motility and the acrosome reaction (fusion of sperm outer acrosomal membrane and plasma membrane and activation of sperm enzymes) has been shown in human serum (Yanagimachi, 1970; Bavister and Morton, 1974), hamster adrenal glands (Bavister et al., 1975) bovine adrenal cortex and medulla (Bavister et al., 1979) and bovine follicular fluid (Yanagimachi, 1969). Liu et al. (1977) suggested that one of the factors in bovine follicular fluid, responsible for inducing the acrosome reaction was albumin.

A recent report by Meizel et al. (1980) indicated that follicular fluid has another factor, the sperm motility factor (SMF), identified as taurine or hypotaurine, which stimulates sperm motility. They showed that there were high levels (range 0.054-1.525 mM) of these two compounds in rabbit uterine and ampullar oviductal fluid, bovine follicular fluid, macaque oviductal fluid (M. fascicularis) and bovine adrenal gland.

The sperm motility stimulating activity of the SMF was enhanced by a cofactor, identified to be the catecholamines, epinephrine or norepinephrine (Bavister et al., 1979). When catecholamines were added to the hamster sperm, the characteristic whiplash flagellar movement (Yanagimachi, 1970) characteristic of capacitated sperm was observed (Garnett and Meizel, 1978). Using α- and β- adrenergic (catecholamine) agonist compounds, phenylephrine and isoproterenol, Garnett et al. (1979) reported

that the ability of hamster sperm to fertilize *in vitro* required both α- and β- adrenergic stimulation of the acrosome reaction. Meizel and Working (1980) demonstrated that the negative isomers of catecholamines were more effective than positive isomers in stimulating hamster sperm acrosome reaction. For instance, the percent acrosome reaction after 4 hours of incubation for negative positive epinephrine and controls were 55, 36, and 10% respectively. The postulated that the catecholamines might be inhibiting phosphodiesterase activity or by acting as a chelator of metal ions indirectly stimulating ATPase activity with consequential activation of acrosome reactions.

A class of compounds called the methyl xanthines, which can inhibit cyclic nucleotide phosphodiesterase activity have been shown to stimulate sperm motility in the bull (Garbers *et al.*, 1971a; Garbers *et al.*, 1971b) the human (Schoenfeld *et al.*, 1975) the golden hamster (Morton and Chang, 1973) and the mouse (Fraser, 1979). The methyl xanthines include caffeine, aminophylline, theophylline, isobutylmethyl xanthine (MIX or IBMX) and pentoxifylline. Phosphodiesterase stimulators, such as imidazole, have an opposite action. When 6 mM caffeine (1,3,7-trimethyl-2, 6 dioxy-purine) was added to bull sperm, a significant increase in motility ($p<0.05$) was observed and by 4 hours the treated sperm had a 55.2% motility rating compared to 27.2% for controls (Garbers *et al.*, 1971a). The respiratory rate was also noted to increase in the treated group (1.98 x endogenous rate) over the controls (1.22 x endogenous rate) in the presence of pyruvate. Human sperm, with an initial motility of 60-80%, also responded with increases in motility when supplemented with 6mM caffeine (72.6 $\pm$ 0.78%) compared to controls (53.1 $\pm$ 1.16%) after 4 hours (Schoenfeld *et al.*, 1975). In addition, caffeine prolonged human sperm motility. This observation was not seen in golden hamster sperm, which, although motility

or the rate of flagellation was increased (35% treated vs. 20% controls after 4 hours at 6 mM caffeine concentration) with caffeine, it did not prevent the rapid decline in motility (Morton and Chang, 1973). When caffeine was tested on a mouse in vitro fertilization system, a higher rate of fertilization was noted for the caffeine treated group (76.7%) compared with controls (32.1%) after 1 hour incubation (Fraser, 1979). The increase in the fertilization rate of the caffeine treated group was attributed to changes in the sperm rather than the oocytes because of the changes in sperm motility patterns observed, and because the majority of the oocytes were fertilized in caffeine-containing medium while the majority of control oocytes were unfertilized. This suggested that cAMP might be a controlling factor in sperm capacitation, since, caffeine will increase intracellular cAMP levels through inhibition of phosphodiesterase activity. Supporting evidence of a cyclic nucleotide involvement came from the fact that sperm have adenyl cyclase systems (Hardman et al., 1971; Casillas and Haskins, 1971) and, at the time of capacitation, a decrease in ATP occurs (Upchurch and Morton, 1972). The effects of cAMP on sperm and fertilization have been extensively studied. Toyoda and Chang (1974) reported shorter penetration times and increased sperm penetration of rat oocytes (90.7%) when sperm were preincubated with 2 mM dbcAMP for 5-6 hours in the presence of 34.8 mM KCl. The controls had 0% penetrated oocytes at 3 hours. They postulated that the process of capacitation involved activation of adenyl cyclase and the addition of exogenous cAMP accelerated this process.

In that same year, Rosado et al. (1974) published a paper providing further evidence of cAMP involvement in the capacitation process. Preincubation of rabbit sperm for 6 hours in 0.1 μM dbcAMP increased the

sperm capacity to fertilize, as shown by the percent of cleaved rabbit oocytes (20.0% tested vs, 3,1% control). Negative control treatment with dbcAMP and follicular fluid, but no sperm, had no cleavage indicating that dbcAMP action was not on the oocytes. Reyes et al. (1978) reported that supplementation of dbcAMP with 2.5 μM calcium and ionophore A23187 in the culture medium increased the percent cleavage from 17% to 44%. Rogers and Garcia (1979) supported the concept of dbcAMP (10 mM) stimulation with guinea pig sperm, (76% treatment vs. 53% controls at 4 hr.). They also reported that as the percent motility was increased, the percent acrosome reaction occurrence declined rapidly (10% treatment vs. 43% controls at 4 hr.) and suggested that during capacitation and the acrosome reaction there is a reduction of intracellular cAMP in the guinea pig sperm. Santos-Sacchi et al. (1980) confirmed these observations. They demonstrated that the guinea pig sperm acrosome reaction could be induced by cGMP and when the sperm adenylate cyclase system was inhibited by Zn^{++} ions (250 μM $Zn\ Cl_2$), the percent of sperm showing the acrosome reaction increased (23.46% treated vs. 0.09% controls). They indicated that this difference in the role of cAMP in the acrosome reaction may be due to the species differences.

In the golden hamster, the addition of 0.1-10 μM dbcAMP stimulated sperm acrosome reactions 2-3-fold over controls at 5 hours (Mrsny and Meizel, 1980). The sperm motility was not different from the controls throughout the incubation.

Studies on human sperm (DeTurner et al., 1973) indicated that 0.6 mM dbcAMP stimulated significantly more forwardly progressive sperm after 4 hours. They suggested that cAMP aids in prolonging human sperm motility. Using multiple exposure photography, Makler et al. (1980) reported that

dbcAMP did not affect human sperm motility. They attributed this lack of stimulation to the high activity of phosphodiesterase in the sperm. Using infrared spectral analysis, Delgado and colleagues (1976) showed that in control human sperm, the acrosomal membranes were in the most stable protein configuration, (the antiparallel β- conformation; "pleated sheets"). When they added 2.04 μM dbcAMP to the sperm, they noted a change in the membrane protein structure towards the less stable, α-helix and random coil forms. They hypothesized that cAMP acts by causing a change in the acrosomal membrane structure from a stable to an unstable form in addition to increasing sperm metabolism and motility.

II. In vitro Fertilization

A review of in vitro fertilization in mammalian species including the cat, dog, pig, sheep, cow, rabbit, mouse, rat, hamster and guinea pig, has been published by Brackett (1979). The historical aspects of in vitro fertilization of primate oocytes was reviewed by Kuehl (1976). The present review will focus on the modern period (1968-1981) of in vitro fertilization in non-human and human primate species.

In vitro fertilization in non-human primates

A. Oocyte maturation in vitro

One of the major problems for in vitro fertilization research is obtaining matured oocytes at the time of insemination. Maturation is defined as the stage when the oocytes are in the metaphase II stage of meiosis division, characterized by the presence of a polar body. It is only when the oocytes are mature that they are ready for sperm penetration. Since most of the oocytes recovered are not matured, they have to be incubated in medium for a certain length of time (in vitro maturation) before they become mature. Early work with primate oocytes at the

dictyate stage, cultured for 20 hours in vitro failed to induce germinal vesicle breakdown and maturation (Edwards, 1962). In a later report, Edwards (1965) noted that it required at least 30 hours of in vitro culture for rhesus monkey oocytes to mature. Many oocytes stopped developing at anaphase I and this was attributed to a medium deficiency of a particular component.

In vitro maturation in the absence of gonadotropins (Edwards, 1965) has been documented. The fertility of oocytes matured in vitro was tested by Suzuki and Mastroianni (1968). They obtained a total of six presumptive fertilized Macaca mulatta oocytes out of thirty-one (19.4%) from the oviducts after a two-step process of 48 hours in vitro maturation and oocyte transfer. Their in vitro maturation procedure yielded 55.3% matured oocytes.

Thompson et al. (1971) compared the ultrastructural changes in M. mulatta oocytes cultured in vitro for 48 hours with in vivo matured oocytes. They found no difference between in vitro and in vivo matured oocytes. The ability of the oocytes to mature depended on pre-existing conditions in the follicle prior to culturing.

Shorter times for in vitro maturation have been reported by Thibault et al. (1976) who stated that M. fascicularis oocytes required only 23-26 hours in vitro culturing to reach metaphase II. Follicles with diameters below 1.4 mm were unable to mature and remained at metaphase I. They suggested that the cumulus cells-oocyte complex responded differently in immature and preovulatory follicles. Interestingly, the in vitro maturation rate of squirrel monkey oocytes from different sized follicles were not significantly different from each other (Kuehl and Dukelow, 1976, 1979).

Smith et al. (1978) reported differences in in vitro maturation rates of M. mulatta oocytes obtained during the breeding (October-January) versus the non-breeding season (April-July). At the end of a 48-hour incubation period, 58% of the oocytes extruded the first polar body in the breeding season group compared with 21% in the non-breeding season group. They postulated that the follicular environment, particularly an increased estrogen level, facilitated subsequent oocyte maturation in vitro during the breeding season. The effect of seasonality on squirrel monkey in vitro oocyte maturation is considered in the present experiments.

B. In vitro fertilization in non-human primates

With the discovery of capacitation (the process the sperm undergoes to acquire the ability to fertilize oocyte) successful in vitro fertilization was accomplished in many species including the primates. In 1972, Kraemer (unpublished) described in vitro fertilization of 5 baboon (Papio cynocephalus) oocytes in Bavister's and Brinsters media with subsequent development of one fertilized oocyte to the six-cell stage. Johnson et al., (1972) first reported attachment of sperm to the zona pellucida when they cultured Saimiri sciureus (squirrel monkey) oocytes in media containing estrone sulfate. Cline et al. (1973; Gould et al., 1973) reported 11 of 22 oocytes fertilized in vitro, with 6 proceeding to the 2-cell stage. They noted the extrusion of the second polar body between 20-24 hr after insemination and cleavage was observed 12 hours later.

Kuehl and Dukelow (1975a; Dukelow and Kuehl, 1975b) reported in vitro fertilization of 32 of 79 squirrel monkey oocytes with some developing to the 4-cell stage. There were no significant differences in fertilization rates using culture medium TC199 or Ham's F-10-based media, but there was a higher maturation and fertilization rate in a chamber-slide culture

system compared with Karrel flask culture dishes.

In an extended study, Kuehl and Dukelow (1979) reported a fertilization rate of 33.5%. A comparison of five different media indicated that TC-199 supplemented with 20% calf serum and 72 μg/ml of pyruvate resulted in the highest maturation and fertilization rates, 56.5% and 73.9% respectively. There were no differences in oocyte recovery between laparotomy (34.4%) and laparoscopy (35.2%) or in subsequent in vitro fertilization (35.7 and 43.3% respectively).

C. In vitro preimplantation stage development in the non-human primate

In vitro and in vivo fertilized embryos of the rabbit (Onuma et al., 1968) mouse (Brinster, 1963; Hsu et al., 1974), swine (Davis and Day, 1978), rhesus monkey (Lewis and Hartman, 1973), baboon (Kraemer, 1972), squirrel monkey (Kuehl and Dukelow, 1975a, 1979; Dukelow, 1981) and human (Rock and Menkin, 1944; Seitz et al., 1971; Edwards and Steptoe, 1975; Soupart and Strong, 1974, 1975; Lopata et al., 1978) have been reported to develop in vitro to stages characteristic of those just prior to implantation.

In the non-human primate, Kraemer (1979) successfully cultured one in vitro fertilized baboon embryo to the six-cell stage by 72 hours in Brinster's medium. In the in vivo environment this was observed 48-72 hours after mating (Hendrickx and Kraemer, 1968). Lewis and Hartman (1933;1941) reported in vitro development of in vivo fertilized rhesus monkey oocytes. They noted first, second, third and fourth cleavage times of 1-1.5, 1.5-2, 2-3, 3-4 days after natural mating respectively. Kuehl and Dukelow (1975a; 1979) observed in vitro development times for first, second and third cleavage of squirrel monkey embryos at 20-40, 46-52 and 52-72 hours after insemination, respectively. Their in vitro time course of development agrees with the human embryo in vitro development time

(Edwards and Steptoe, 1975). They also reported further development of eight-cell in vitro fertilized squirrel monkey embryos to the sixteen-cell stage in the pseudopregnant rabbit oviduct (Kuehl and Dukelow, 1977). In other species, the components necessary for in vitro development have been implicated to be pyruvate and palmitic acid for rabbit embryos (Kane, 1979), glucose for the rabbit blastocyst (Frindhandler et al., 1967), pyruvate and lactate for the mouse (Brinster, 1965a; 1965b), pyruvate for early stages up to 8-cells and lactate for stages up to the blastocyst in the mouse (Quinn and Wales, 1973), and three factors, found in human cord serum that promoted development of the mouse blastocyst to the early somite stage (Hsu, 1980). Bovine serum albumin supported 4-cell porcine embryos to the blastocyst stage and pyruvate inhibited this development (Davis and Day, 1978). Glucose was required for the bovine blastocyst (Renard et al., 1980). Recently Pope et al. (1980) published a paper describing successful culture of in vivo fertilized baboon embryos in CMRL-1066 medium supplemented with 20% heat-inactivated fetal calf serum or human cord serum. Embryos at the 8 to 16 cell stages developed to the blastocyst stage after 5-6 days culture, attached to the culture dish (2 days later) and formation of the primary yolk sac occurred. Recovered blastocysts hatched in vitro after 2-3 days in culture. Currently there are no published reports of in vitro fertilized oocytes developing to the advanced pre-implantation stages in culture.

III. In vitro Fertilization in Humans

A. In vitro maturation of human oocytes

There were early reports of in vitro studies done on human oocytes (Pincus and Saunders, 1939; Rock and Menkin, 1944; Mayashi, 1963). Edwards observed germinal vesicles in the oocytes after 20 hours of culture (1962) and reported in vitro maturation (resumption of meiosis up to Metaphase II)

after 40-48 hours of culture (Edwards, 1965a). When inseminated, sperm was observed in the perivitelline space (1965a). Germinal vesicle breakdown, Metaphase I and Metaphase II occurred 25, 28-35 and 36-43 hours after oocyte recovery, respectively (Edwards, 1965b), Jagiello et al. (1968) reported observing oocyte maturation 47-50 hours after HCG treatment. In an extended study, Edwards et al. (1969) reported an in vitro maturation rate of 57.5% in follicular fluid, supplemented with either Hank's, Brinster's or Bavister's medium. When a chemically defined medium, Kregs-Ringer bicarbonate with added glucose, lactate and pyruvate, was used for in vitro maturation of oocytes, Kennedy and Donohue (1969) obtained a 15 percent maturation rate. Using Ham's F-10 medium, they reported that in vitro maturation was higher in oocytes with cumulus cells (13%) compared with cumulus free-oocytes (6%). They suggested that the cumulus cells supplied factors necessary for oocyte maturation.

Based on in vitro observation of oocyte maturation, Steptoe and Edwards (1970) aspirated follicles by means of laparoscopy, 29-31 hours after HCG treatment. The recovery rate was about 33%. Three out of ninety-seven recovered oocytes were in the Metaphase II stage. Nine other oocytes reached this stage after 3-18 hours of culture in follicular fluid with 50% Bavister's medium. Laparoscopic examination confirmed the process of ovulation at 37-38 hours after HCG (Edwards, 1973).

Ultrastructural analysis was done on different stages of in vitro matured oocytes (Zamboni et al. 1972). Their results indicated a 38.2% maturation rate after 37 hours or less of culture, and 67.4% after 40-53 hours. Comparison of the oocytes matured in vitro and in vivo showed no differences ultrastructurally, and that in vitro maturation did not induce significant anomalies.

Soupart and Morgenstern (1973) using Bavister's medium (25%) and follicular fluid (75%), evaluated oocytes maturing with exogenous FSH, LH and HCG. They reported a higher maturation rate in the presence of exogenous hormones (57.1%) compared to controls (28.0%) but the difference was not significant. Soupart and Strong (1974; 1975) described a method using hormones for in vitro maturation of immature oocytes. This consisted of culturing the oocytes in Ham's F-10 medium with added 17β- estradiol for 4 hours and then culturing in 17α-hydroxy-progesterone for 44 hours. Shea et al. (1975), however, did not observe any differences in in vitro maturation of oocytes in the presence of progesterone (36.6%) compared with untreated controls (39.0%). They did notice that the highest maturation rate came from patients 34-39 years of age.

Thibault (1977) reported a difference between oocytes matured in vivo and in vitro. With in vitro maturation, the male nucleus penetrating the oocyte did not dissociate and swell immediately, as it does with in vivo culture. There were also grains of chromatin in the male pronucleus not seen under natural culture conditions. He suggested that the discrepancy might be due to the late appearance of a male pronucleus growth factor in the intact follicle.

In a review of oocyte maturation, Moor and Warner (1979) summarised the events during maturation. The synthesis of heterogenous and ribosomal RNA occurred until germinal vesicle breakdown. Mitochondrial RNA was continuously synthesized. There appeared to be short-lived tyrosine-rich proteins synthesized to regulate early meiotic events. The maturing oocyte needed pyruvate or oxaloacetate and oxygen. The membrane junction between the oolemma and the follicle cells became disrupted and there was an increase in K^+ permeability and increased amino acid transport across

the membrane.

B. In vitro fertilization of human oocyte

Preliminary attempts at in vitro fertilization of human oocytes were reported prior to 1969 (Menkin and Rock, 1944; Hayashi, 1963; Edwards et al. 1966). Edwards et al. (1969) successfully fertilized seven of thirty-four mature oocytes in vitro. Five of the fertilized oocytes, however, were polyspermic. In another study, Bavister et al. (1969) reported observing sperm midpiece and tail in four of six fertilized oocytes 10.5-24 hours after insemination. The most successful medium for in vitro culture was Ham's F-10 supplemented with human or calf serum (Edwards and Fowler, 1970).

The use of sperm, pre-incubated in the rhesus monkey uterus for 4 hours, resulted in in vitro fertilization of 8 of 50 oocytes (Seitz et al. 1971). The fertilized oocytes were in various stages of development (2-12 cells) by 72 hours of culture. They supplemented Ham's F-10 medium with estrone sulfate. The incidence of sperm penetration was significantly increased, when exogenous FSH, LH and HCG were included in the medium (Soupart and Morgenstern, 1973). In the presence of these hormones, they obtained a fertilization rate of 12.5% compared to 0% for controls. They suggested that the sperm required capacitation, probably initiated by the hormones, and that the hormones acted through the follicle cells. Electron microscopy revealed the sperm tail in the oocytes (Soupart and Strong, 1974). The oocytes did not develop further. The time of observation of pronuclei was 11.5 hours after insemination (Edwards, 1973).

Early reports of transferring in vitro fertilized embryos to recipients lack details and were discounted by scientists. Edwards and Steptoe (1976) reported a right tubal ectopic pregnancy after reimplantation of an in vitro developed blastocyst. The embryo did not survive due to persistent bleeding.

On July 25, 1978, Steptoe and Edwards in England reported the birth of a baby girl resulting from transfer of an in vitro fertilized ovum. The oocyte had been recovered on November 10, 1977, fertilized in vitro and developed to the eight-cell stage. This presented strong support for the validity of in vitro fertilization systems. Subsequently a second child (male) was born in England from this procedure. A total of five pregnancies have occurred there. In India, another in vitro fertilized baby girl, born on December 2, 1978 was reported by two research workers, Mukkarjee and Bhattacharya (Blandau, 1980). This embryo was allegedly frozen and thawed prior to transfer. Details of this work have not been documented nor has the work been confirmed.

The experiments on in vitro fertilization of oocytes, recovered using a suction aspiration device (Lopata et al. 1974), suggested that human sperm were capacitated in vitro, after 3-4 hours (Lopata et al. 1978a). These workers reported a 55.6% fertilization rate. Decondensation of the sperm chomatin in the vitellus required three hours followed by pronuclei formation in an additional three hours. Cleavage of the embryos in culture was observed up to the 8-cell stage and viable embryos were transferred back into the oocyte donor (Lopata et al. 1978b). Pregnancy was achieved from transfer of an in vitro fertilized 8-cell embryo (Lopata et al. 1980) and later reports indicated that the baby had been born and several other pregnancies are still in progress.

C. In vitro development of preimplantation human embryos

The use of Ham's F-10 medium supplemented with human or calf serum has facilitated development of the in vitro fertilized embryos in culture (Edwards and Fowler, 1970; Seitz et al. 1971; Edwards, 1973a). The first, second and third cleavage times were 18-39, 38-46 and 51-62 hours of incubation, respectively (Edwards and Fowler, 1970). The fourth cleavage

time, the morulla and blastocyst stages were later reported to be 63-85, 111-135 and 123-147 hours of incubation, respectively (Edwards, 1973a; 1973b). Seitz et al. (1971) reported cleavage of eight in vitro fertilized oocytes to the 12-cell stage by 72 hours of culture. Staining of embryos showed chromatin in the blastomeres. Parthenogenic cleavage was not observed in the controls. Human blastocysts do not exhibit rhythmic hatching pulsations when cultured in vitro (Edwards, 1973b). The hatching phenomenon has not been observed in vitro. Embryos cleaved abnormally when high osmotic pressure medium was used (Edwards, 1973a).

Human cord serum or human embryo extract has been used as supplement to the Ham's F-10 medium (Lopata et al. 1978). Using such media, they showed that preovulatory oocytes had a higher cleavage rate (55.6%) than nonovulatory oocytes (0.0%).

Extensive developmental studies of cultured human embryos have not been done for ethical and moral reasons. This emphasizes the value of non-human primate models for such developmental studies.

IV. In vitro fertilization in the golden hamster

The in vitro fertilization of golden hamsters was first reported by Yanagimachi and Chang (1962; 1964). Superovulation was induced with pregnant mare serum gonadotropin and at least, a 44 hour interval (Fleming and Yanagimachi, 1980) before HCG treatment. The extension of the interval to 72 hours resulted in increased fertilization with no increase in chromosomal abnormalities (Mizoguchi and Dukelow, 1980). The medium used by Yanagimachi and Chang (1964) was Tyrode's solution or a mixture of TC199 and glycine. The observed slower penetration of oocytes and lower fertilization rates with epididymal sperm compared with uterine incubated sperm. Embryos fertilized in vitro developed only to the two-cell stage. A period of 4 hours for sperm capacitation was noted by Barros and Austin

(1967). Niwa et al. (1980) recently noted that capacitation required the presence of follicular cell components. In vivo experiments showed that follicular contents had no effect on fertilization (Moore and Bedford, 1978). Cornett and Meizel (1978) reported that catecholamines stimulated capacitation and the acrosome reaction. The stimulation by catecholamines was potentiated by a sperm motility factor from the adrenal glands (Bavister et al. 1979). A high rate of fertilization was noted when catecholamines were added to the cultures (Cornett et al. 1979). Since catecholamines elevate intracellular cAMP, cAMP was added to the in vitro cultures but was observed to decrease the acrosome reaction and fertilization (Rogers and Garcia, 1979). In a recent report, this observation was contradicted when added cAMP significantly stimulated the acrosome reaction (Mrsny and Meizel, 1980). They suggested an involvement of cyclic nucleotides in hamster sperm capacitation and the acrosome reaction. The present investigation examined the role of cyclic nucleotides in hamster in vitro fertilization.

Materials and Methods

Animals Used

Adult squirrel monkeys (Saimiri sciureus) of Bolivian origin (Primate Imports Corp., Port Washington, New York) were maintained indoors with a 12 hr: 12 hr Light:dark cycle at 21 ± 2°C. During the summer months (June to October) the animals were housed in large gang cages outdoors (Jarosz and Dukelow, 1976). They were fed a commercial monkey feed and fresh water ad libitum.

Ovulation Induction Regimen

The follicular oocyte development and ovulation regimen consisted of treating the female squirrel monkeys with four daily i.m. injections of follicle stimulating hormone (1 mg, FSH-P, Burns-Biotic Laboratories, Inc., Omaha, Nebraska) and a single i.m. injection of human chorionic gonadotropin (250 iu, HCG, APL ® Ayerst Laboratories, Montreal) on the fourth day (Dukelow, 1970; 1979). During the anovulatory season (July-Sept.) five days of FSH injections (rather than four) are given (Kuehl and Dukelow, 1975b) followed by HCG treatment.

Laparoscopic Recovery of Squirrel Monkey Oocytes

The laparoscopic procedure for oocyte recovery has been previously described (Dukelow et al., 1971; Dukelow and Ariga, 1976). This procedure consisted of anesthetizing the monkey with sodium pentobarbital (27 mg/kg body weight per adult animal, i.p.). A small midline incision was made and the trocar-cannula inserted. The trocar was then removed and the laparoscope (4 mm diameter, Karl Storz Co.) inserted. A 25 gauge needle and 1 ml tuberculin syringe were used to move the fimbria aside to expose the ovaries. The follicles were punctured using the needle and the oocytes aspirated into 0.05 ml of medium in the syringe. The oocytes were then placed into sterile 8-chamber tissue culture slides (Lab-Tek Products,

Napierville, IL) and incubated in a 37°C moist atmosphere of 5% CO_2 in air. The cultures were periodically observed with an inverted microscope and the medium replaced daily using a drawn-out Pasteur pipette. Cultures contaminated with red blood cells were washed by flushing 0.2 ml of medium into each chamber. The oocytes, being heavier, sunk to the chamber bottom, and 0.2 ml of the diluted medium was aspirated carefully and discarded. The final volume in all chambers was adjusted to 0.25 ml. During observation of the cultures, a chamber-slide incubator (Clinical Scientific Equipment Co., Melrose Park, IL) held the cultures at 37°C.

Semen Collection

The male monkey was held in a V-shaped restrainer (Kuehl and Dukelow, 1973) and short pulses of current (frequency 120 pulses per second; duration 5 msec) were delivered to a rectal probe. The voltage was gradually increased and decreased in a rhythmic fashion every 3 seconds. The semen, as a white coagulum, was collected and diluted in medium and held at 37°C for 5-10 minutes. The ovum-containing cultures were inseminated with 0.05 ml of sperm suspension 36 to 37 h after HCG injection of the females (10^5 to 10^6 sperm/ml). Semen volume and sperm motility based on visual estimation were recorded. Aliquots of the sperm suspension was used to study the effect of culture additives on sperm motility. In the cyclic nucleotide experiments described in a later section the compounds were added at the time of insemination to the culture. They were also added in the replacement medium daily.

Culture Medium

The medium used was TC-199 (with 25 mM HEPES Buffer, Earle's Salts and L-Glutamine, GIBCO Laboratories, New York) supplemented with 20% heat inactivated GG-free fetal bovine serum (GIBCO Laboratories), 1 mM pyruvate (Sigma Chemical Co., St. Louis), 100 Units per ml Penicillin-Streptomycin

initially, replaced by 100 µg per ml Gentamicin ® (Schering Corp.) later, and 1 unit per ml heparin. All media were sterilized by passing through a 0.45 µm Millex filter (Millipore Corp.) and stored in 10 ml vacutainer tubes at 4°C. Fresh medium was prepared every 3 weeks and unused medium discarded.

In the cyclic nucleotide experiments, the treated cultures received either 1 or 10 µM final concentration of dibutyryl cAMP (Sigma Chemical Co., St. Louis, Catalog D-0627, Lot 98C-7430) or 10 µM dbcGMP (Sigma Chemical Co., Catalog D-3510, Lot 109C-7630) dissolved in medium.

The sperm motility factor, SMF (Bavister et al. 1976) was prepared from homogenized female hamster adrenal glands and the SMF was heat-inactivated and sterilized with the millipore filter (0.24 mg/chamber). The concentration of DL-norepinephrine (Sigma Chemical Co., Catalog A 7256, Lot 20C-3880) and L-epinephrine (Sigma Chemical Co., Catalog E-4250, Lot 108C-0269) used in the SMF experiments were 20 µM each (Cornett et al., 1979). The continuous flow culture system was set up as shown in figure 1. An automatic syringe dispenser (Sage Instruments, Inc., Model 234-7) dispensed 37°C medium at the rate of 0.4 ml per 6 hr into the culture chamber and as the level of medium rose it was aspirated into a flask through a vacuum system.

In the circulating medium experiments, cultures were slowly shaken at 37°C on a variable speed rotator table (Clay-Adams, Inc.) set at the lowest speed (105 revolutions/min). The cultures were observed over a 3 day period.

Visual Assessment of Sperm Motility

The sperm aliquots (0.02-0.05 ml) from the diluted ejaculate was placed into the chambers containing treated and control media. The

chamber was then mounted on the inverted microscope and the chamber slide incubator placed over the chambers to maintain the 37°C temperature. The percent motile sperm (number of sperm swimming in the field of vision) was then visually assessed at 5 different locations in each treatment chamber and the average recorded.

The sperm motility was estimated at intervals of 0, 30, 60, 120 minutes and so on, up to 10 hours.

In assessing the ejaculated monkey sperm for each trial, the initial motility, the degree of progressive motility, the percentage of immature sperm (as denoted by cytoplasmic droplets), and the concentration in each chamber were noted.

Criteria of Maturation and Fertilization

At intervals of approximately 24 hr the cultures were examined and the stage of development noted. The presence of a polar body indicated that the oocytes was mature. The criteria for fertilization were as follows:

(1) two or more polar bodies in the perivitelline space (occasionally 2 pronuclei are also observed, but at other times were difficult to see due to surrounding cumulus cells)

(2) two or more polar bodies and two or more equal sized blastomeres by 24 hr after insemination

(3) verification of two or more sets of diploid chromosomes through Giemsa staining (Mizoguchi and Dukelow, 1980)

(4) observation of the sperm tail or midpiece within the cytoplasm.

When an oocyte had one or more of the above criteria, it was designated as fertilized.

Hamster Gamete Recovery

The hamster in vitro system was designed as a preliminary screening system for testing variables. Adult female golden hamsters (Mesocricetus

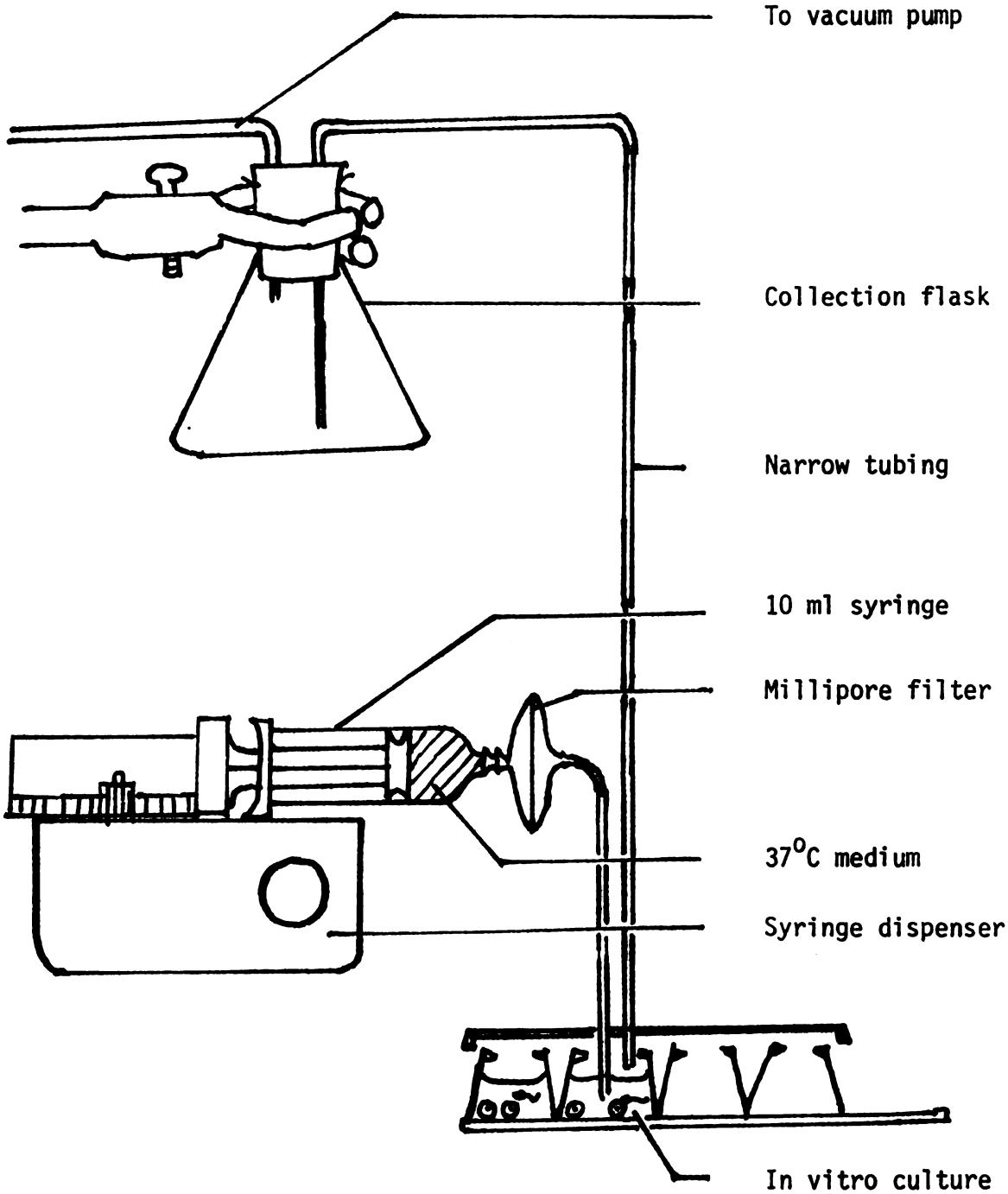

Figure 1. Apparatus for the continuous flow experiment in hamster in vitro fertilization system.

auratus, Charles River Labs., Wilmington, MA, 80-160 g, 10-20 weeks of age) kept at 14 hr:10 hr (L:D) cycle, were superovulated using the system of Mizoguchi and Dukelow (1980) with an ip. injection of 30 i.u. pregnant mares serum (PMS) at 800 hr on Day 4 (estrus). The oocytes are flushed out of the fimbriated end of the oviduct with medium with a 30 ga needle on Day 5 at 800 hr and the oocytes cultured in the chamber slides at 37°C under moist 5% CO_2 in air mixture. The male hamsters (100-195 g) were sacrificed and sperm collected from minced epididymis tissue. The cultures were inseminated (about 10^6 sperm/ml) and observed later for evidence of fertilization.

Criteria for Fertilization in Hamster In Vitro System

The oocytes were examined for fertilization 24 hours post insemination. Fertilization was said to occur if (a) there were 2 polar bodies and 2 pronuclei (b) 2 polar bodies and 1 pronuclei, the second pronuclei having not been formed yet (c) 2 pronuclei and 1 polar body and (d) more than 2 polar bodies and /or more than 2 pronuclei (polyspermic fertilization). The progress of the cultures were assessed again 48 hours post insemination. The number of atretic, matured and fertilized oocytes were recovered in each trial.

Statistical Analysis of Data

The Student t-test was used to evaluate differences between values. Paired t-test was used for the evaluation of sperm motility. In the efficacy of recovery trials, two-way analysis was performed. All data were computed using the Hewlett Packard 41C statistical packet.

Results

Squirrel Monkey In Vitro Fertilization System: Technical Parameters

A. Seasonality and Fertility of Male Squirrel Monkeys

To better understand the effects of season on male fertility, the data on the males collected throughout the year were analyzed. Analysis (t-test) of the results (Table 1) indicated no significant differences in the fertilization rates in the four seasons or among the 5 males. The fertilization rates during the breeding season of squirrel monkeys (winter) and in the anovulatory season (summer) were 69.4% and 50.0% respectively. The mean sperm concentration of the 5 male monkeys was 35.1 $\pm$ 18.0 ($X10^5$) sperm/ml. The natural logarithm of the mean sperm concentration of the five males appears to be linearly related to the fertilization rate, over a period of one year for each of the five males with correlation coefficient of 0.8307.

Seasonal Maturation of Oocytes

The high fertilization rates around spring time noted in Table 1 might be explained by the high maturation rate in vitro of the squirrel monkey oocytes at this season (Figure 2). The highest percentage of ova maturing in vitro was in April with an 85.7% maturation rate (12/14). The lowest month was in October with 0% maturation (0/3). The mean rate of oocyte maturation was 49.6 $\pm$ 7.0% (one standard error). The data were based on squirrel monkey follicular oocytes recovered 15-16 hr after HCG treatment and incubated over a period of 3-5 days and thus represents in vitro maturation rates. The data for each month was taken from all in vitro trials done in that particular month.

In Vitro Oocyte Maturation Time

The data presented in Figure 3, show the approximate time the

Table 1. Seasonal variation in fertility of male squirrel monkeys.

Male	No. of oocytes fertilized/matured oocytes (%)				Total No. of oocytes fertilized/ matured oocytes (%)	Mean sperm concentration (10^6 sperm/ml) ± s.e.m.	Number of ejaculate samples
	Winter (Jan-Mar)	Spring (Apr-Jun)	Summer (Jul-Sep)	Fall (Oct-Dec)			
B1610	18/29(62.1)	3/6(50.0)	1/1(100.0)	1/2(50.0)	23/38(60.5)	30.0 ± 8.6	21
B1443	-	3/7(42.9)	2/2(100.0)	0/2(0.0)	5/11(45.5)	14.8 ± 8.7	4
B1621	2/2(100.0)	8/13(61.5)	1/3(33.3)	-	11/18(61.1)	76.0 ± 60.0	8
B1609	5/5(100.0)	10/18(55.6)	1/3(33.3)	-	16/26(61.5)	44.8 ± 8.4	6
B1619	-	1/2(50.0)	0/1(0.0)	-	1/3(33.3)	9.8 ± 4.5	3
Total	25/36(69.4)	25/46(54.4)	5/10(50.0)	1/4(25.0)	56/96(58.3)		42
						Mean: (± s.e.m.) 35.1 ± 18.0	

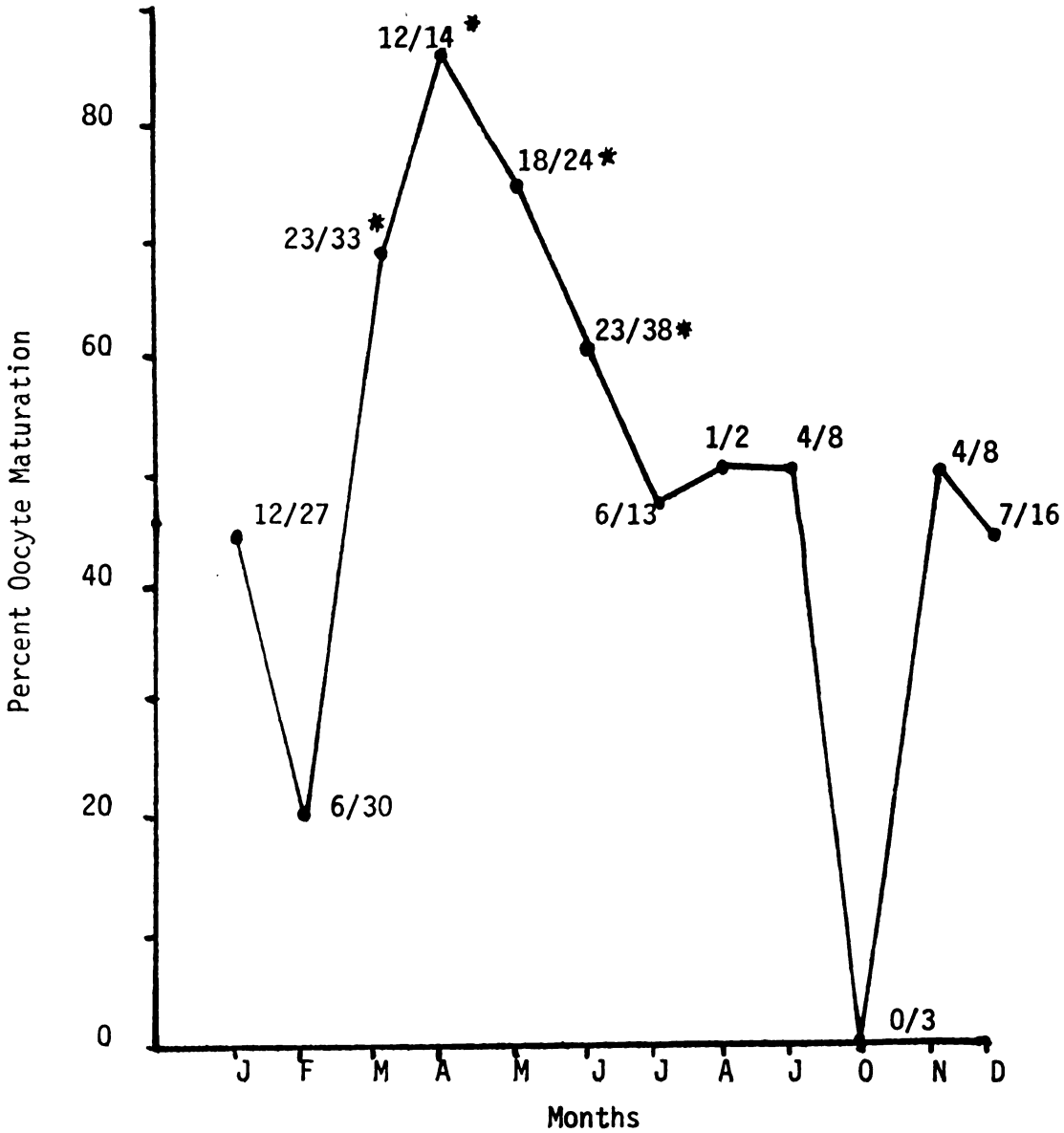

Figure 2. Seasonal variation in squirrel monkey oocyte maturation in vitro. Ratios refer to number of matured oocytes/ total no. of oocytes. * Difference ($P<0.05$).

recovered oocyte extruded the first polar body (maturation). Between 21 to 25 hours after laparoscopic recovery, the highest percentage of oocytes were observed to be mature 28.2% (22/78). The maturation of oocytes in culture occurred between 21 and 35 hours. These data came from a series of trials over a six-month period from December to May.

The Effect of Cumulus Cells on In Vitro Fertilization

It has been reported that cumulus cells act indirectly to inhibit oocyte maturation (Hillënjo et al., 1979). In the squirrel monkey in vitro fertilization system, the effects of the corona radiata and cumulus cells are presented in Table 2. The results indicate that the oocyte with corona radiata cells had a greater maturation rate than oocytes without these cells ($P<0.05$). The lowest maturation rate was found in oocytes without corona radiata cells which had a rate of 51.2%. Studies done in bovine in vitro fertilization systems also confirmed that the presence of corona radiata and cumulus cells significantly increased maturation of oocytes (Fukui and Sakuma, 1980). The mean maturation rate of squirrel monkey oocytes in vitro was 70.3 $\pm$ 4.3% (s.e.m.). The incidence of atresia of the oocyte was significantly higher ($P<0.005$) in the no-corona cells group compared to the rest of the groups.

The in vitro fertilization rate of the oocyte-corona cells complex groups were not significantly different from each other (two-tailed, t-test). Fertilization was independent of the presence of corona radiata and cumulus cells. The mean fertilization rate for the 6 groups of oocytes was 54.8 $\pm$ 6.1 (s.e.m.). The mean time when 2 polar bodies were observed in the oocyte groups was also not significantly different. The mean of all these values was 31.4 $\pm$ 10.1 hours after insemination. The earliest time recorded for observing 2 polar bodies was 2.8 hours after insemination.

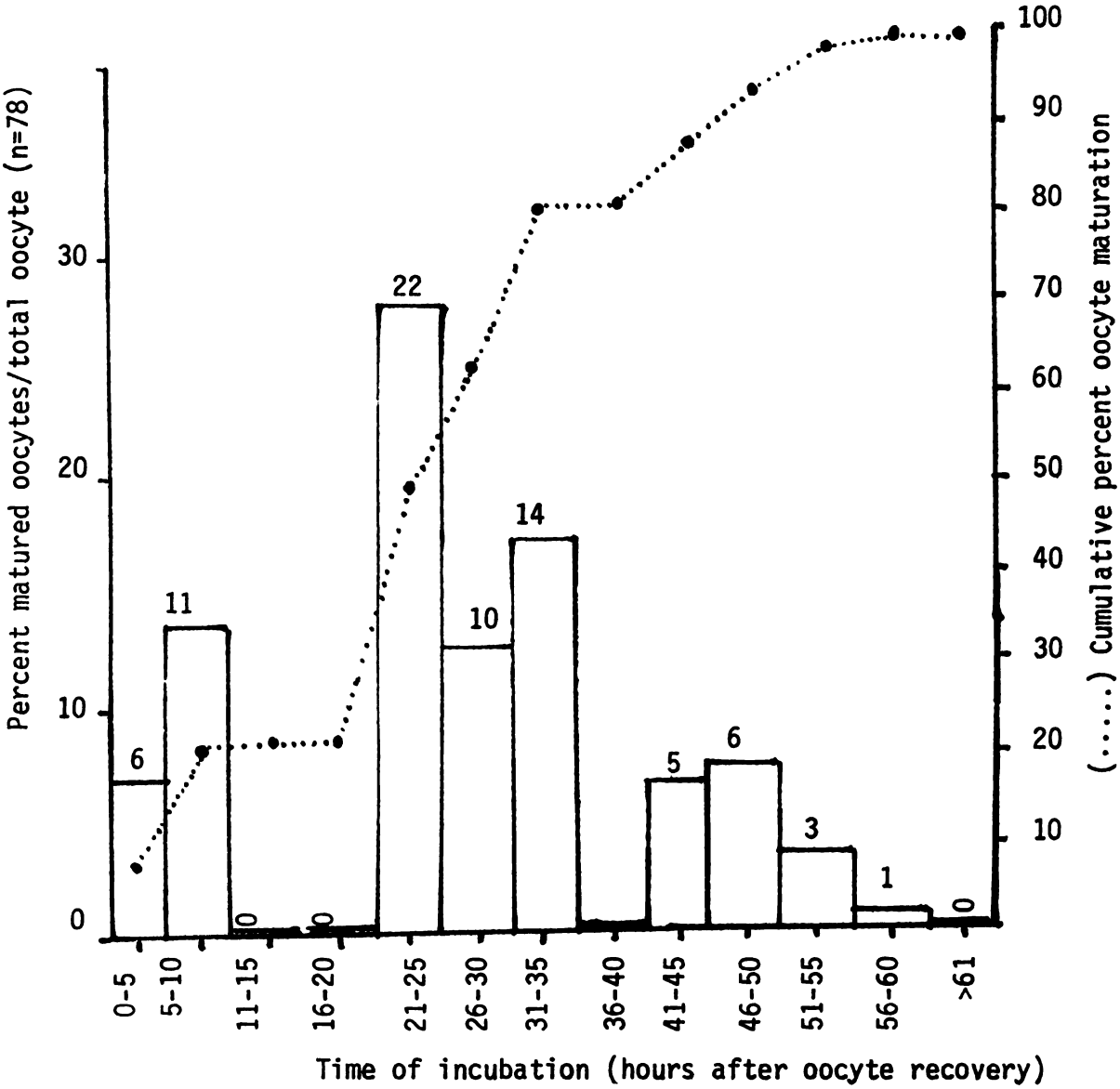

Figure 3. Squirrel monkey oocyte maturation time in vitro. The highest number of oocytes were observed to be matured at 21-25 hours after recovery.

Table 2. The influence of cumulus cells on squirrel monkey in vitro fertilization system.

Amount of corona radiata cells covering oocyte	Total no. of oocytes	No. of matured oocytes (%)	No. of atretic oocytes (%)	No. of fertilized oocytes (%)	Mean time 2pb was observed and range (hr post insemination)
No. corona radiata cells	43	22 (51.2)[a]	15 (34.9)[c]	13 (59.1)	26.5 (6.0-55.5)
One quarter covered with corona radiata cells	17	12 (70.6)[b]	1 (5.9)[d]	9 (75.0)	24.2 (6.8-52.0)
Half covered with corona radiata cells	23	17 (73.9)[b]	2 (8.7)[d]	7 (41.2)	30.9 (2.8-73.0)
Three quarters covered with corona radiata cells	30	22 (73.3)[b]	2 (6.7)[d]	14 (63.6)	19.6 (7.0-36.0)
Completely covered with corona radiata cells	18	14 (77.8)[b]	1 (5.6)[d]	7 (50.0)	43.80 (11.0-75.5)
Covered with corona radiata and cumulus oophorus cells	20	15 (75.0)[b]	1 (5.0)[d]	6 (4.00)	43.4 (72.0-120.0)

[a,b] Values with different superscripts are significantly different ($P<0.05$)

[c,d] Values with different superscripts are significantly different ($P<0.05$)

Table 3 shows the effect of corona radiata and cumulus cells on the development of fertilized oocytes. The highest rate of cleavage to the 2-cell stage was found in the no-corona radiata cells and the three quarters-covered group ($P<0.10$). The mean cleavage rate of the 6 groups was 28.6 $\pm$ 9.2%. The mean observed time of first cleavage was 33.8 $\pm$ 10.6 hr. The 3-cell stage took 73.4 hr. The completed second cleavage took 62.8 hour and the observed fourth cleavage at 75.0 hour. The time for third cleavage was not observed in this series of trials.

Laparoscopic Recovery of Squirrel Monkey Oocytes

The efficacy of oocyte recovery is presented in Table 4. The total number of oocytes recovered in 21 trials was 222 from 541 aspirations (41.0%). Two-way analysis of variance for the left and right ovary indicated insignificant interaction between follicle size and follicular development. If an ovary had poor follicular response to the ovulation regimen, the follicles were generally small. Conversely, if the development was excellent, the follicles tended to be large (>2.0 mm). When the recovery data for the left ovary was compared to the right ovary, a significant difference ($P<0.005$) was noted between them. There was a higher rate of recovery from the right ovary compared to the left ovary (48.2 $\pm$ 2.6% vs 37.1 $\pm$ 3.4%).

Follicular Fluid Effects on the In Vitro Fertilization System

The influence of follicular fluid on the in vitro maturation and fertilization rates of squirrel monkey oocytes was studied in cultures incubated for three days. The results indicate that the follicular fluid volume tested at the 0.0 (control), 0.01, 0.02 and >0.03 ml resulted in maturation rates of 85.7% (6/7), 66.7% (10/15), 83.3% (5/6) and 42.9% (6/14) respectively. There were no significant differences in these rates.

Table 3. Effect of corona radiata cells on the in vitro development of squirrel monkey embryos.

Amount of corona radiata cells on oocyte	No. of cleaved oocytes/fertilized oocytes (%)	Number of development embryos (cell stage)	Observation time (hr after insemination) — 2 cell (mean)	3 cell	4 cell	16 cell
No. corona radiata cells	7/13 (53.9)[a]	1 (4); 1 (16)	40.9	-	52.5	75.0
One quarter covered with corona cells	1/9 (11.1)[b]	0	58.0	-	-	-
Half covered with corona cells	0/7 (0.0)[b]	0	0.0	-	-	-
Three quarters covered with corona cells	6/14 (42.9)[a]	1 (3); 1 (4)	54.1	58.0	73.0	-
Completely covered with corona cells	1/7 (14.3)	0	10.0	-	-	-
Covered with corona and cumulus cells	1/6 (16.7)	1 (3)	39.8	88.3	-	-
Total	16/56 (28.6)	2 (3); 2 (4) 1 (16)	33.8	73.4	62.8	75.0

a,b Values in the same column with different superscripts are different ($P<0.10$)

Table 4. Efficiency of laparoscopic recovery of squirrel monkey follicular oocytes from follicles of different sizes.

Ovary follicular development	Left Ovary			Right Ovary		
	Follicle size[a]			Follicle size[a]		
	1.0 mm	1.0-2.0 mm	2.0-3.0 mm	1.0 mm	1.0-2.0 mm	2.0-3.0
Poor	11/20[b] (55.0)	none	none	17/33 (51.1)	none	none
Moderate	21/48 (43.8)	7/20 (35.0)	none	10/26 (38.5)	9/18 (50.0)	none
Good	23/62 (37.1)	44/127 (34.7)	17/48 (35.4)	10/21 (47.6)	28/51 (45.9)	11/20 (55.0)
Excellent	none	4/17 (23.5)	3/8 (37.5)	none	none	7/12 (58.3)

[a] All follicle sizes in millimeters.

[b] Oocytes/no. of follicles aspirated (percent)

Table 5. Fertilization and development of squirrel monkey oocytes at different recovery and insemination times.

Group	Time of oocyte recovery (hours post HCG)	Time of insemination (hours post recovery)	No. of matured oocytes/total oocytes (%)	No. of Fertilized oocytes/ matured oocytes (%)	No. of 2-cell embryos/ fertilized oocytes (%)	Further development
1	16	21	35/66 (53.0)	16/35 (45.7)	2/16 (12.5)	one 4-cell
2	36	24	24/43 (55.8)	14/24 (58.3)	3/14 (21.4)	-
3	36	13	10/22 (45.5)	7/10 (70.0)	2/7 (28.6)	onw 3-cell

The fertilization rates were 16.7% (1/6), 50.0% (5/10), 80.0% (4/5) and 33.3% (2/6) respectively. The presence of 0.02 ml follicular fluid had a significantly higher ($P<0.05$) fertilization rate than controls. Development of the fertilized oocytes was observed only in the 0.01 ml group (2/5, 40.0%) and the 0.02 ml group (2/4, 50.0%). These trials were conducted from April to August.

Fertilization and Development at Different Recovery and Insemination Times

Some researchers have reported that oocytes have a critical period of RNA synthesis at the final period of maturation in response to LH or HCG injection (Davidson et al., 1964; Edwards et al., 1969). The results of the present squirrel monkey studies (Table 5) indicated that the in vitro maturation and fertilization and cleavage rates were not significantly different between oocytes recovered at 16 and 36 hours after HCG. The in vitro maturation period or the time of insemination after recovery tested at 13, 21 and 24 hours did not show any significant differences in the maturation and fertilization or cleavage rates. Oocytes recovered 36 hours and inseminated 49 hours after HCG had the highest rate of cleavage (28.6%). One 3-cell embryo was observed in this last group. These trials were conducted from June to October.

Squirrel Monkey Sperm Motility Studies

Sperm motility factor, SMF, (Bavister et al., 1976) and its cofactors, catecholamines (Cornett and Meizel, 1978; Bavister et al., 1979) have been implicated as a sperm motility stimulant. These factors were tested on squirrel monkey sperm and the results are presented in Figure 4. The results indicated that 20 μM DL-norepinephrine significantly stimulated (maintained motility above controls) ($P<0.05$) sperm motility by 10% over a period of 10.5 hours. L-epinephrine also stimulated sperm motility but this effect was insignificant and was observed only after 2 hours incubation.

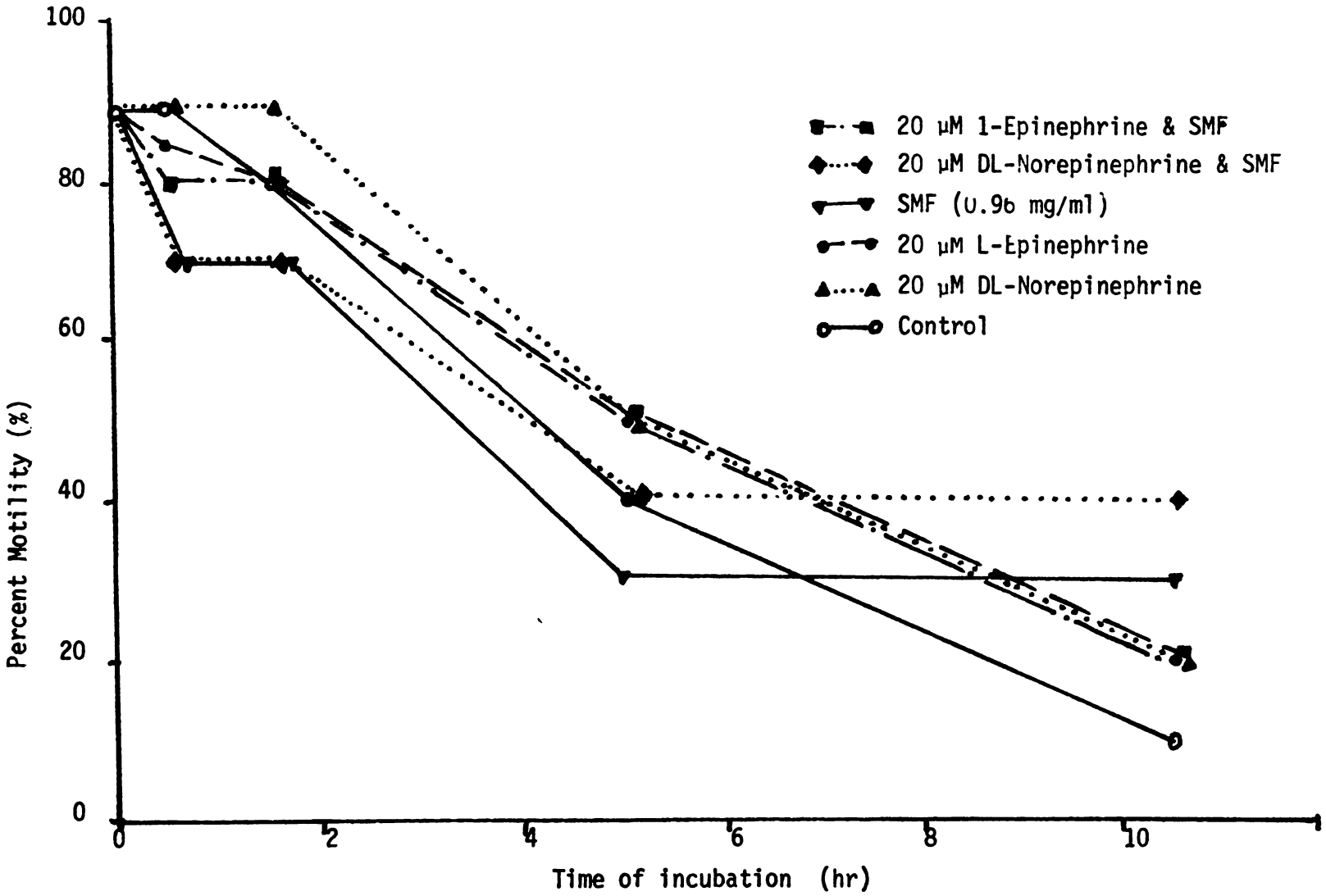

Figure 4. The effects of Norepinephrine, Epinephrine and SMF on squirrel monkey sperm motility. (Each point is the mean of 5 observations).

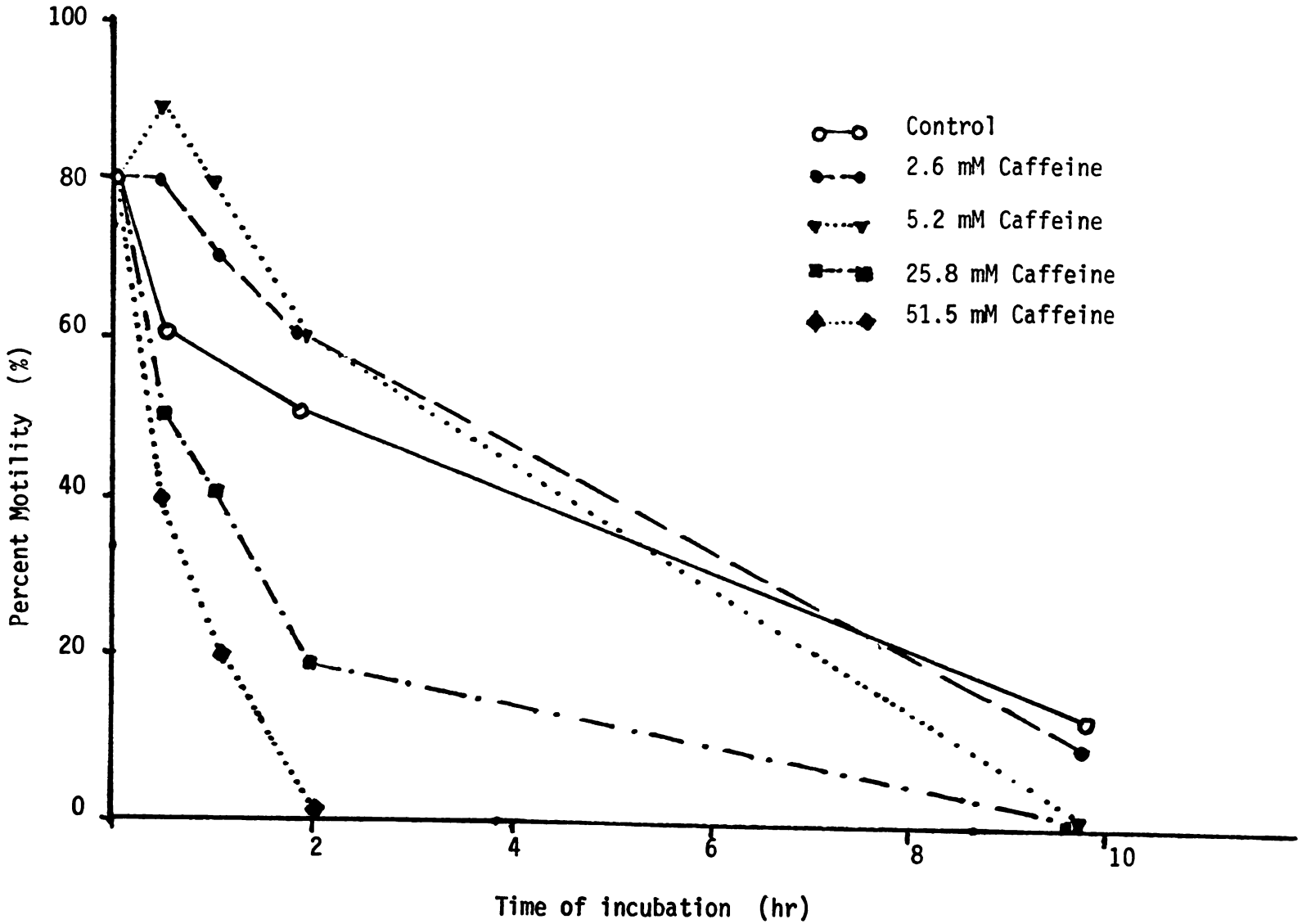

Figure 5. Effects of Caffeine on monkey sperm motility. (Each point is the mean of 5 observations).

The adrenal extract (SMF) inhibited motility up to 5 hours in culture and when this was added to the catecholamines, a reduction of the stimulated effect was noted with norepinephrine while it did not have an effect on the stimulation by epinephrine. At 10.5 hours all treatment groups had higher sperm motility than the controls but only the norepinephrine-SMF group had a significant ($P<0.05$) motility sustaining effect. By 22.0 hours all cultures lacked sperm motility except the three cultures containing SMF.

Since catecholamines are phosphodiesterase inhibitors (Goren and Rosen, 1972), another compound with this property, caffeine, reported to stimulate ejaculated bovine sperm motility (Garbers et al., 1971), was also tested on squirrel monkey sperm. The results are shown in Figure 5. At the 2.6 mM and 5.2 mM caffeine level, there were up to 30% significant increases ($P<0.05$) in sperm motility compared to the controls for a period of 3 hours. When 25.8 mM or 51.5 mM caffeine was added to the cultures, the sperm motility declined rapidly with total cessation by 3 hours. Caffeine did not prolong the sperm motility.

One effect of the phosphodiesterase inhibitor is to raise intracellular cAMP levels in the sperm. To study the direct cause of stimulated sperm motility dibutyryl cAMP was tested on squirrel monkey sperm. This derivative of cAMP was used because it penetrates cell membranes much more readily than cAMP (Posternak et al., 1962) and is more resistant to phosphodiesterase activity (Kaukel et al., 1972). The motility of the sperm was significantly increased ($P<0.10$) by 10 to 20% when 0.05 μM or 1.2 μM dbcAMP was added over a minimal period of 3.5 hours. (see Figure 6). The 0.7 μM dbcAMP level stimulated sperm motility at the 3.5 hour observation point. The period of stimulated activity appeared to be similar to low concentration caffeine addition ($r=0.9234$). All treatments were the

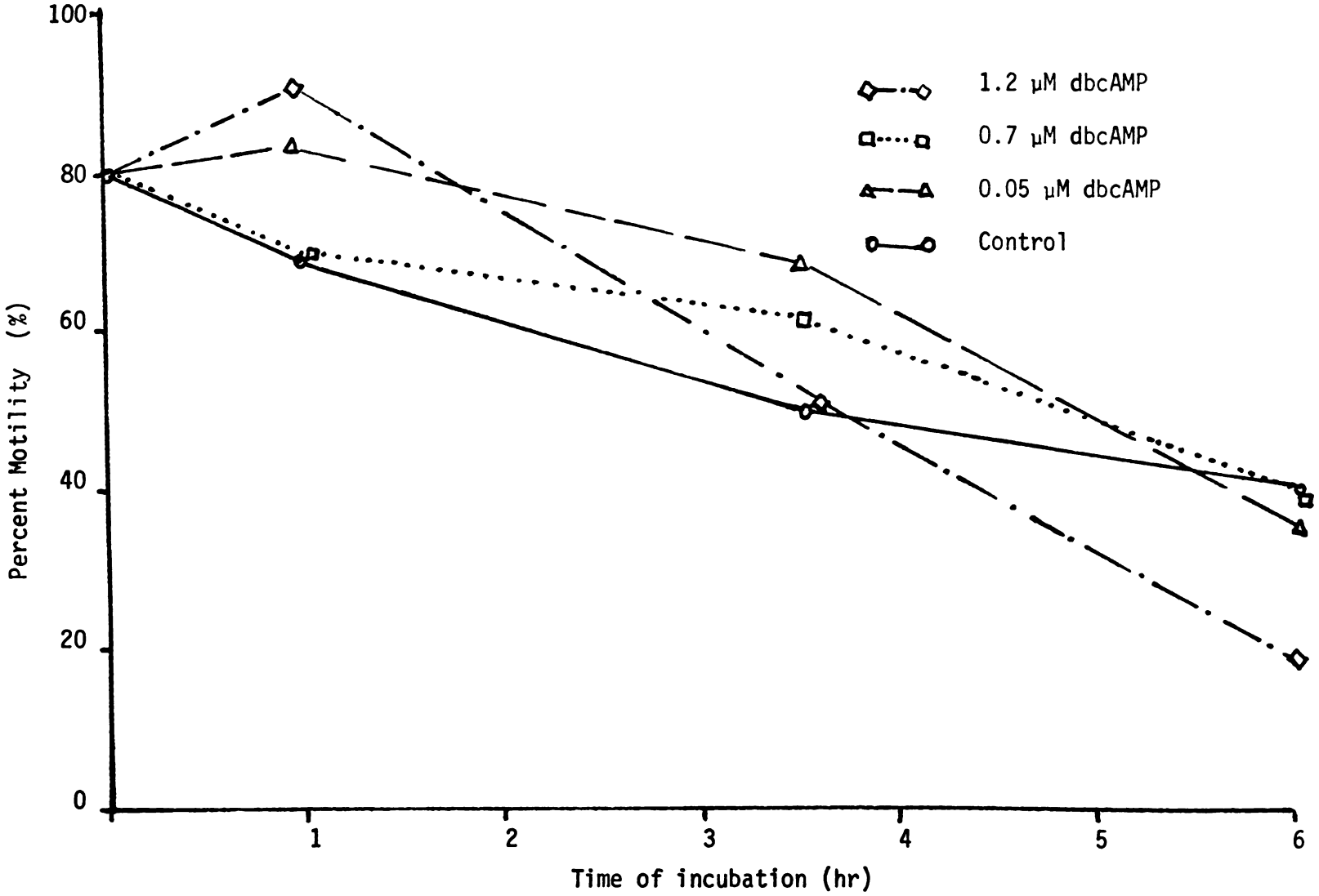

Figure 6. Stimulatory effect of dibutyryl cAMP on squirrel monkey motility. (Each point is mean of 5 observations).

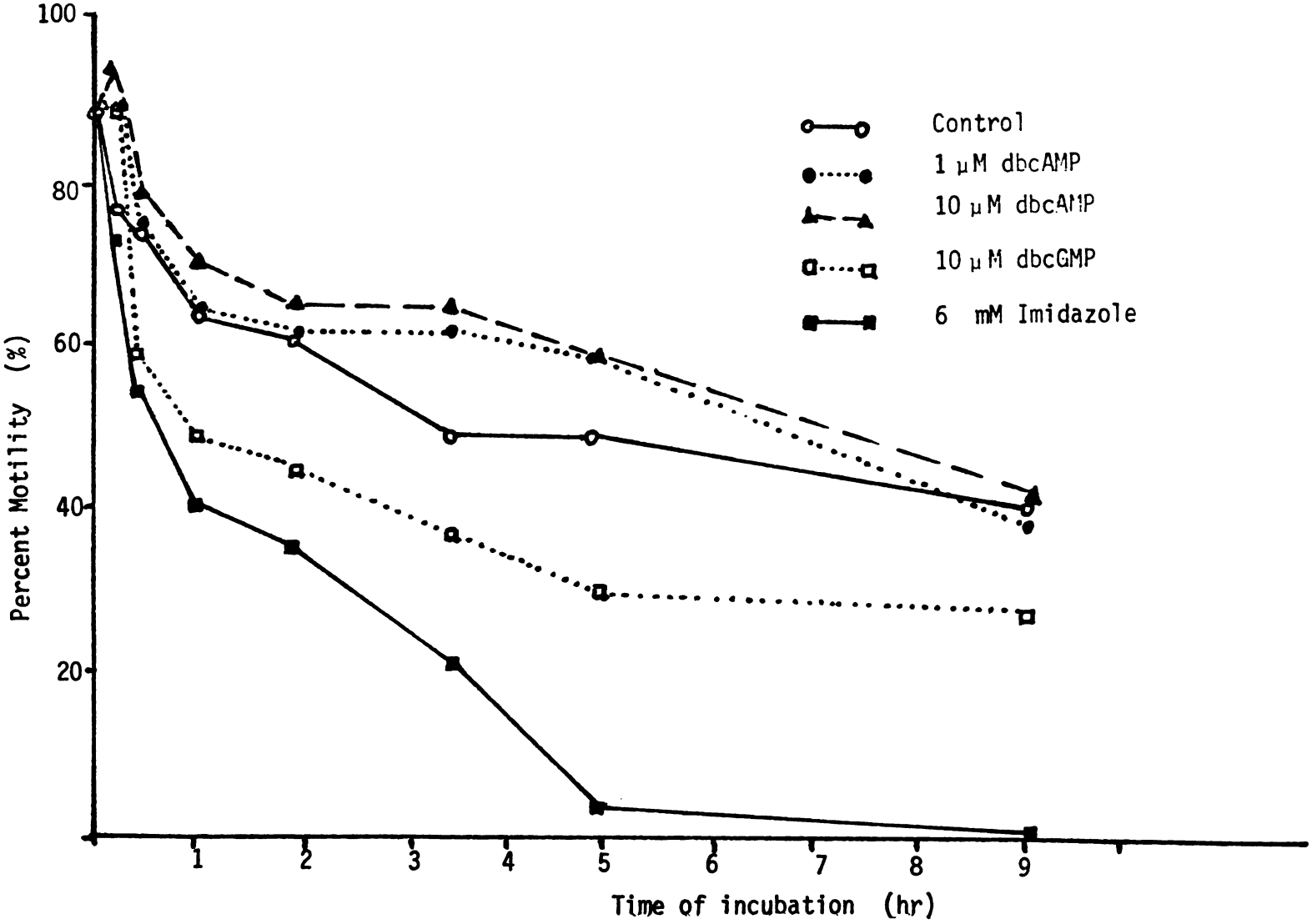

Figure 7. The effect of dbcAMP, dbcGMP and imidazole on squirrel monkey sperm motility. (Each point is the mean of 5 observations).

same or below control cultures after 6 hours of incubation.

The results of adding two phosphodiesterase stimulators, dbcGMP and imidazole, which lower intracellular cAMP levels are presented in Figure 7. A significant decrease ($P<0.05$) in sperm motility of about 10% was observed with the 10 μM dbcGMP treatment. The 6 mM imidazole treatment significantly decreased ($P<0.05$) sperm motility up to 40%, compared to controls, with total cessation of activity at 9 hr incubation. The addition of 1 μM and 10 μM dbcAMP significantly stimulated ($P<0.05$) sperm motility up to 10% over the controls and this effect was sustained for at least 5 hours. When dbcAMP was added after the sperm had been incubated for 4.5 hours, a burst of stimulated motility ($P<0.05$) was observed (see Figure 8). This increased motility (10%) was still observed after 12 hours of incubation compared to the controls and the dbcAMP-added at time 0 group.

In Vitro Fertilization Studies

Preliminary trials were conducted with hamsters, prior to extending the studies to squirrel monkeys. The results of the effect of circulating medium (cultures put on rotating tables) on hamster in vitro fertilization, is presented in Table 6. The maturation rates of the control and shaken cultures were not significantly different because the oocytes were matured in vivo prior to testing the treatment. Cultures that were shaken had a significantly lower ($P<0.01$) fertilization rate (25.9%) compared to control (70.8%). Further, the incidence of polyspermy was significantly greater ($P<0.01$) for shaken cultures. After 48 hours of incubation, the percentage of degenerated oocytes was significantly higher ($P<0.01$) in the shaken cultures (78.8%) compared to controls (7.7%). Owing to the fact that shaken cultures had lower fertilization rates, this method of culture was not tested with the squirrel monkey in vitro fertilization system.

A continuous medium flow culture system, tried on hamster oocytes and

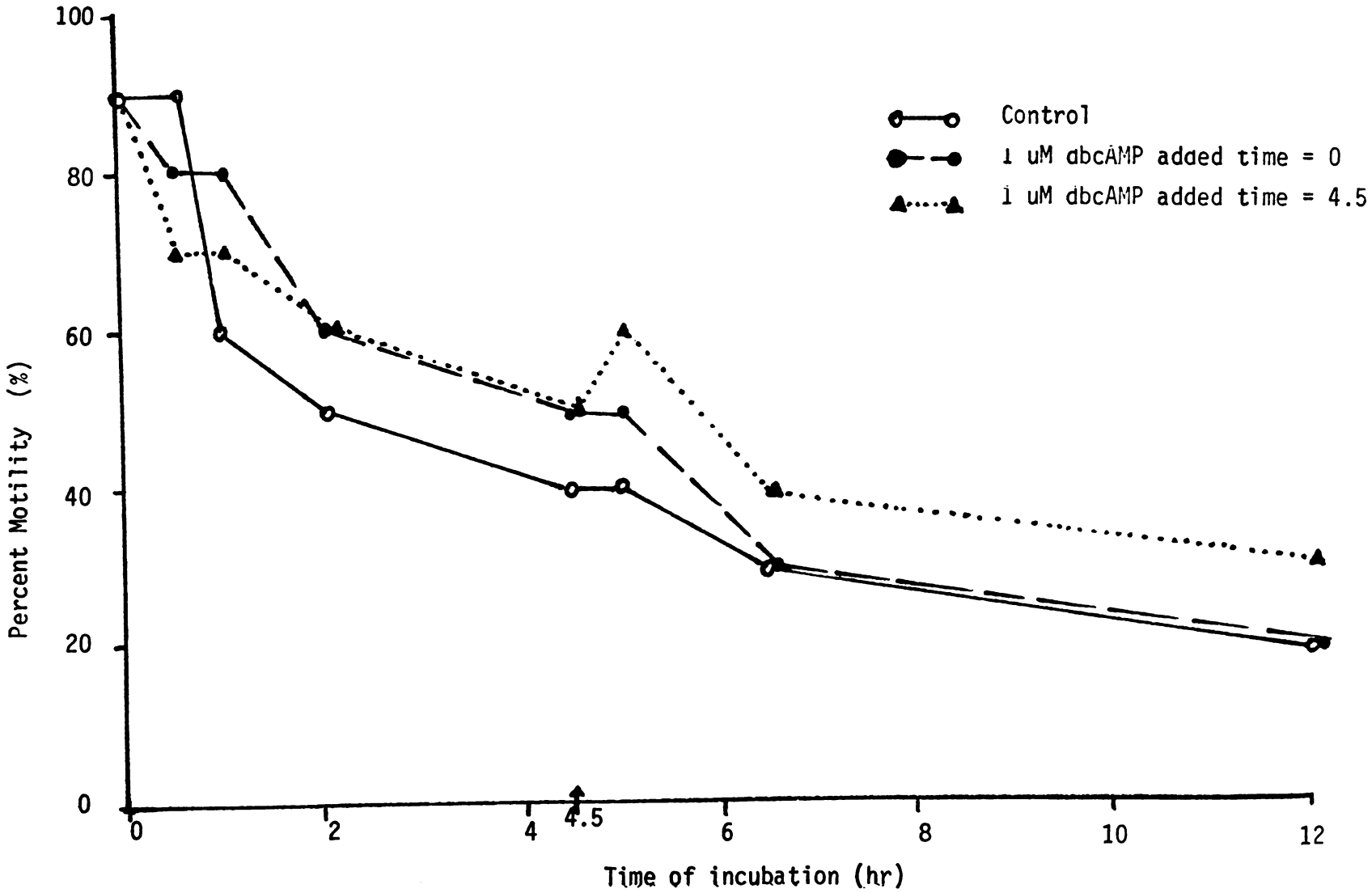

Figure 8. The effect of adding dbcAMP to 4½ hour old squirrel monkey sperm. (Each point is the mean of 5 observations).

Table 6. Effect of circulating medium (shaking) on hamster in vitro fertilization.

Treatment	No. of oocytes recovered	No. of matured oocytes (%)	No. of fertilized oocytes (%)	No. of polyspermic oocytes (%)	No. of degenerated oocytes (%)
Control culture	26	24 (92.3)	17 (70.8)	0 (0.0)	2 (7.7)
Shaken culture	33	27 (81.8)	7 (25.9)[a]	6 (85.7)[a]	26 (78.8)[a]

[a] Significant difference ($P<0.01$) from control cultures.

Table 7. The effect of continuous flow on hamster in vitro fertilization

Treatment	No. of trials	No. of matured oocytes/total oocytes (%)	No. of fertilized oocytes/ matured oocytes (%)	No. of polyspermic oocytes/ fertilized oocytes (%)	No. of degenerated total oocytes (%)
Controls	2	32/37 (86.5)	19/32 (59.4)	2/19 (10.5)	11/37 (29.7)
Continuous flow	2	35/46 (76.1)	24/35 (68.6)	2/24 (8.3)	4/46 (8.7)[a]

[a] Significant difference ($P<0.01$) from control culture

sperm, yielded the results shown in Table 7. The maturation rates of the control and treated groups were not significantly different. The fertilization rate and the incidence of polyspermy were also not significantly different between groups. The percent degeneration, however, was significantly higher ($P<0.01$) for the control group.

Studies to provide a means of waste removal and medium replacement were also done in the squirrel monkey in vitro fertilization system. The results, shown in Table 8, indicated that there were no significant differences in maturation, fertilization or first cleavage rate between the control and the medium-replacement group.

Gentamicin®, a broad spectrum antibiotic widely used in tissue culture systems was evaluated for its efficacy in the squirrel monkey in vitro fertilization system. The results (Table 9) showed no significant differences between regularly used penicillin-streptomycin and Gentamicin® in terms of maturation, fertilization and cleavage rates.

The effect of adding cyclic nucleotides to the hamster cultures is presented in Table 10. There were no significant differences in the maturation rate and incidence of polyspermy. However, the fertilization rate of the 1 μM dbcGMP group (93.3%) was significantly greater ($P<0.05$) than the control (57.1%) and 1 μM dbcAMP group (42.9%). The addition of dbcAMP had no effect on the fertilization rate. A greater number of oocytes with 2 polar bodies and 2 pronuclei were observed in the dbcGMP treatment group.

Table 11 shows the results of adding cyclic nucleotides to the squirrel monkey in vitro fertilization system. Addition of 1 and 10 μM dbcAMP enhanced the fertilization rate above controls (90.2, 90.0 vs. 60.0% respectively, $P<0.01$). The addition of 10 μM dbcGMP resulted in

Table 8. Effect of medium replacement on maturation and fertilization in vitro in the squirrel monkey.

Treatment[a]	No. of matured oocytes/total oocytes (%)	No. of fertilized oocytes/matured oocytes (%)	No. of 2-cell embryos/fertilized oocytes (%)	Number of developed embryos (cell stage)
Control	33/58 (56.9)	18/33 (54.5)	7/18 (38.9)	1 (3); 1 (4) 1 (8)
Medium replacement	51/75 (68.0)	33/51 (64.7)	7/33 (21.2)	1 (3)

[a] The experiment consisted of 16 trials.

Table 9. Effect of Gentamicin® on the squirrel monkey in vitro fertilization system

Treatment	No. of matured oocytes/ total oocytes (%)	No. of fertilized oocytes/ matured oocytes (%)	No. of 2-cell embryos/ fertilized oocytes (%)	No. of developed embryos (cell stage)
Control[a]	37/70 (52.9)	20/37 (54.1)	4/20 (20.0)	2 (3)
100 µg/ml Gentamicin	15/31 (48.4)	7/15 (46.7)	2/7 (28.6)	1 (4)

[a] Control cultures contained 100 Units/ml Penicillin-streptomycin

Table 10. The effect of cyclic nucleotides on hamster in vitro fertilization

Treatment	No. of matured oocytes/ total oocytes (%)	No. of fertilized oocytes/ matured oocytes (%)	No. of polyspermic oocytes/ fertilized oocytes (%)	No. of fertilized oocytes with 2 pb and 2 pn[a]
Control	7/7 (100.0)	4/7 (57.1)[b]	0/4 (0.0)	1/7 (14.3)
1 μM dbcAMP	7/7 (100.0)	3/7 (42.9)[b]	0/3 (0.0)	1/7 (14.3)
1 μM dbcGMP	15/15 (100.0)	14/15 (93.3)[c]	2/14 (14.3)	7/15 (46.7)

[a] 2 polar bodies and 2 pronuclei.

[b,c] Values with different superscripts indicate significant difference ($P<0.05$).

Table 11. Effects of dbcAMP and dbcGMP on fertilization and cleavage of squirrel monkey ova fertilized in vitro.

Treatment	No. of trials	No. of mature oocytes	No. of fertilized oocytes (%)	No. of oocytes cleaved to 2-cell stage (%)	Final developed stage
Control	32	85	51 (60.0)[a]	14 (27.5)	1 (8-cell)
1 uM dbcAMP	16	41	37 (90.2)[b]	12 (32.4)	12 (2-cell)
10 uM dbcAMP	14	30	27 (90.0)[b]	6 (22.2)	2 (4-cell)
Control	11	18	13 (72.2)[c]	3 (23.1)	3 (2-cell)
10 uM dbcGMP	11	43	22 (51.2)[d]	7 (3i.8)	1 (8-cell)

[a,b] Values with different superscripts indicate significant difference (P 0.01)

[c,d] Values with different superscripts indicate significant difference (P 0.10)

a lower fertilization rate ($P<0.10$) compared to controls (51.2 vs. 72.7% respectively). The rates of first cleavage for control, dbcAMP and dbcGMP groups were not significantly different from each other. dbcAMP and dbcGMP did not inhibit two-cell embryos to develop further in the cultures.

Discussion

Squirrel monkeys exhibit strong reproductive seasonality in the wild. They mate from June to September (Rosenblum, 1968). In captivity, there is a shift of the mating season to the winter months, January to April. Seasonal effects were not observed on the fertilizability of oocytes, in vitro. In the present studies where follicular development was induced with gonadotropins, the winter months had the highest fertilization rate, but this was not statistically significant.

The effect of seasonality on the in vitro maturation of oocytes was pointed out by Smith et al. (1978). Rhesus monkey oocytes had significantly higher in vitro maturation rates during the breeding season (50-60%) compared to the non-breeding season (20-30%). The present data indicate that the squirrel monkeys have a significantly higher in vitro maturation period in the months of March to June (60.5-85.7%) compared to a mean maturation rate of 37.4% for the remaining months. The present colony of squirrel monkeys has been in captivity for about two years and the observed high maturation rate in spring may reflect a transition in the shift to the winter months.

The in vitro maturation time required for human oocyte has been reported to be 24 hours (Seitz et al. 1971). The in vitro maturation time for squirrel monkey oocytes was about 24 hours in culture (Kuehl, 1974). The present study indicates that the percent of in vitro matured squirrel monkey oocytes increases slowly from 0 to 20 hours incubation indicating some of the oocytes may have begun maturation prior to the HCG injection. At 21 to 25 hours incubation, there is a high level of maturation. It is for this reason insemination of the cultures was done 21-22 hours after oocyte recovery. The role for corona radiata and

cumulus cells in promoting maturation of oocytes have been reported (Fukui and Sakuma, 1980), and this effect was confirmed on squirrel monkey oocytes. When the mean maturation rate of all oocytes with at least some corona cells were compared to the group of no-corona cells oocytes, a significant difference was noted ($P<0.05$). Corona radiata and cumulus cells increase the maturation rate and decrease the atresia rate possibly by supplying the oocyte with steroids or with some maturation factor. The corona radiata and cumulus cells, however, do not play a role in fertilization. Curiously, high first cleavage rates were observed in the no-corona radiata cells and three-quarters covered with corona radiata cells groups.

The percentage of oocytes recovered from follicles varies from 32.0% (Steptoe and Edwards, 1970) to 41.0% (Lopata et al., 1974). The recovery efficiency (number of oocytes recovered/follicle aspirated) for squirrel monkey oocytes is at a rate of 41.0%. There was a higher recovery rate for the right ovary. This technical difference may be due to the fact that this researcher is right-handed, facilitating recovery from that ovary.

The percentage of oocytes recovered increased with increasing size of the follicle (Edwards, 1973). Lopata et al. (1974) noted a high recovery rate from medium-sized follicles. Kuehl and Dukelow (1979) reported the highest recovery rate came from large sized follicles. In contrast, the data presented in Table 4 indicates no differences in the recovery rate among different sized follicles.

An oocyte maturation inhibiting factor found in follicular fluid (Tsafiri et al., 1976) has been reported to inhibit meiosis. Inhibition of oocyte maturation was not observed when varying amounts of follicular fluid was incubated with the oocytes. It is possible that higher titers of the

follicular fluid are needed to observe inhibition or that once the oocyte is removed from the follicle, meiosis begins. Fertilization events, however, were influenced by the volume of follicular fluid used with the optimum volume being 0.02 ml in a total culture volume of 0.25 ml. Some researchers have suggested that follicular fluid contains components (Yanagimachi, 1970) that trigger capacitation and the acrosome reaction (Yanagimachi and Chang, 1964; Gwatkin and Andersen, 1969) and the present data supports this contention.

When the oocyte recovery time and insemination time were varied, no significant differences were noted in maturation and fertilization rates. Edwards et al. (1969) pointed out the necessity of in vivo maturation for RNA synthesis but the development of in vitro fertilized squirrel monkey embryos appears to be independent of in vivo or in vitro maturation time.

The sperm motility studies were conducted with the intention of studying compounds that stimulate motility and possibly, capacitation and the acrosome reaction. This in turn would lead to higher rates of fertilization. A sperm motility factor and catecholamines stimulated hamster sperm motility (Bavister et al. 1976; Cornett and Meizel, 1978). DL-norepinephrine significantly stimulated squirrel monkey sperm motility above the controls. L-epinephrine did not have any significant effect and the hamster sperm motility factor inhibited monkey sperm motility in contrast to hamster sperm. The data showed that squirrel monkey sperm have predominantly beta-adrenergic receptors involved with motility. Further experiments using receptor blockers will provide further information on sperm receptors.

Caffeine has been reported to stimulate bovine (Garbers et al. 1971) murine (Fraser, 1979) and human (Makler et al. 1980) sperm. The present work shows that squirrel monkey sperm is also stimulated by caffeine at

low concentrations. Since, both, catecholamines and caffeine, increase intracellular cAMP levels, a direct effect of cAMP was tested on squirrel monkey sperm. The results indicated that the cAMP is involved in sperm motility. To test this hypothesis further, two phosphodiesterase stimulators, dbcGMP and imidazole, were added to reduce cAMP levels. The sperm motility decreased with these compounds, providing further support for the hypothesis. When cAMP was added at a later time, the sperm motility increased, indicating reversibility. It has been suggested that cAMP acts by destabilizing the sperm plasma membrane (Delgado et al. 1976), by acting on sperm glycolytic enzymes to speed up glycolysis (Haskins, 1973) or by activating a protein kinase involved in contraction of fibrils (Bailey and Villar-Palasi, 1971).

Three methods were tried to provide a means of supplying fresh medium to the oocytes in culture. The first method, the circulating medium method, had an adverse effect on the hamster oocytes in culture. It is possible that the minimum speed of the rotating table was too rapid since it was twice the speed used by New (1973). The continuous medium flow system tested on the hamster in vitro fertilization system showed no differences in maturation or fertilization rates compared to controls. The percent degenerated oocytes was significantly lower in the continuous flow system.

The method for replacing medium on a daily basis proved useful not only in the removal of spent medium and red blood cells that hinder observation, but also in that it had no adverse effect on fertilization. Similarly, the use of Gentamicin® as an antibiotic in the culture medium, was possible without adverse effects. Of significance is the effect of cyclic nucleotides on hamster and squirrel monkey in vitro fertilization. It has been reported (Rogers and Garcia, 1978) that 10 mM dbcAMP treated hamster oocytes had lower fertilization rates (3.2%) compared to controls

(76.1%). The present data indicate that dbcAMP has no effect on hamster in vitro fertilization. When dbcGMP was added, there was a significant increase in the fertilization rate which may be indirectly related to lowered intracellular cAMP levels. Cyclic GMP has been reported to induce guinea pig sperm acrosome reaction by increasing calcium ion influx (Santos-Sacchi et al. 1980). The hamster sperm, like the guinea pig sperm appears to be activated by cGMP, instead of cAMP.

In contrast to hamster studies, squirrel monkey sperm motility is inhibited by cGMP and this is manifested by the lowered fertilization rate. The addition of cAMP shortened the capacitation time, in one case, to 45 minutes, and increased the fertilization rate by about 30.0% over controls. The action of cAMP is on the sperm since it increases the sperm motility. This view is confirmed by work done in the mouse (Fraser, 1979)

The differences noted in the control of hamster sperm activation (by cGMP) and squirrel monkey sperm activation (by cAMP) may be attributed to a species difference. It has been reported that cGMP activates guinea pig sperm acrosome reactions (Santos-Sacchi et al., 1980) while cAMP activates rat (Toyoda and Chang, 1974), rabbit (Rosado et al., 1974) and human (DeTurner et al., 1973) sperm. Apparently, the type of nucleotide that causes sperm activation depends on the species under study.

The cleavage rates of the in-vitro fertilized oocytes were not affected by cAMP or cGMP, although the percent cleavage in the cGMP group was higher. It has been reported that low levels of cAMP promote microfilament-mediated contraction of cell processes and microtubule disassembly (Willingham and Pastan, 1975). Pienkowski (1980) indicated that cAMP decreased germ cell layers in mouse blastocysts. The preimplantation development of squirrel monkey embryos do not seem to be affected by cAMP and 4-cell stages have been reached.

Summary and Conclusions

The squirrel monkey in vitro fertilization system provides an excellent non-human primate model for the elucidation of the intricacies of primate reproductive physiology. The studies conducted produced the following results:

1) There was no observable seasonality in fertilizability of oocytes. The rate of maturation was significantly higher in the months of March to June. In vitro maturation was observed 21-25 hours after incubation.

2) The groups with varying degrees of corona radiata and cumulus cells covering the oocyte were not different in maturation or fertilization rates. The mean maturation rate of all corona radiata cells-covered oocytes, however, was higher than the no-corona radiata cells group. Follicular fluid had no effects on oocyte in vitro maturation but affected the fertilization rate with an optimum level of 0.02 ml/0.25 total fluid volume.

3) The mean time for observing 2 polar bodies was 31.4 $\pm$ 10.1 hours after insemination. The earliest recorded time was 2.8 hours. The mean first cleavage time was 33.8 $\pm$ 10.6 hours.

4) The mean oocyte recovery rate from follicles aspirated was 41.0%. The right ovary had a higher recovery rate than the left (48.2 vs. 37.1%). There were no differences in recovery rates from follicles of different sizes.

5) The time of recovery (16 and 36 hours after HCG) and the time of insemination (13, 21 and 24 hours after recovery) did not affect maturation, fertilization or cleavage rates of squirrel monkey oocytes.

6) DL-norepinephrine significantly stimulated sperm motility indicating a predominence of beta-adrenergic receptors. The L-epinephrine did

not affect the squirrel monkey sperm motility significantly while the hamster Sperm Motility Factor decreased motility.

7) Caffeine and dbcAMP stimulated sperm motility while imidazole and dbcGMP inhibited motility.

8) The circulating medium system had an adverse effect on hamster oocytes. The continuous flow system had no effect on the maturation and fertilization rates of hamster oocytes. Replacement of medium daily and Gentamicin® addition did not affect squirrel monkey oocytes.

9) The addition of dbcGMP increased the in vitro fertilization rate of hamsters. dbcAMP had no effect on hamster in vitro fertilization rates.

10) The addition of dbcAMP increased the squirrel monkey in vitro fertilization rate by 30 percent while dbcGMP lowered the fertilization rate. An involvement of cAMP in the capacitation and acrosome reaction of the squirrel monkey sperm is postulated. The mean time for observing 2 polar bodies in the dbcAMP treated group (N=23) was 18.3 $\pm$ 2.4 hours after insemination (in contrast to controls, 31.4 $\pm$ 10.1 hours). The mean first cleavage time in the dbcAMP treated group was 37.8 $\pm$ 7.4 hours.

References

Bailey, C. and C. Villar-Palasi. 1971. Cyclic AMP-dependent phosphorylation of troponin. Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 30, 552.

Barros, C. and C. R. Austin. 1967. In vitro fertilization and the sperm acrosome reaction in the hamster. J. Exp. Zool. 166, 317-324.

Bavister, B. D., R. G. Edwards and P. C. Steptoe. 1969. Identification of the midpiece and tail of the spermatozoan during fertilization of human eggs in vitro. J. Reprod. Fert. 20, 159-160.

Bavister, B. D. and D. B. Morton. 1974. Separation of human serum components capable of inducing the acrosome reaction in hamster spermatozoa. J. Reprod. Fert. 40, 495-498.

Bavister, B. D., R. Yanagimachi and R. J. Teichman. 1976. Capacitation of hamster spermatozoa with adrenal gland extracts. Biol. Reprod. 14, 219-221.

Bavister, B. D., A. F. Chan and P. C. Fu. 1979. Catecholamine requirement for hamster sperm motility in vitro. J. Reprod. Fert. 56, 507-513.

Blandau, R. J. 1980. In vitro fertilization and embryo transfer. Fertil. Steril. 33, 3-11.

Brackett, B. G. 1979. In vitro fertilization and its assessment with embryo culture. Animal Reproduction. BARC Symposium No. 3. Edited by Harold Hawks. Allenheld, Osmun, Montclair, pp. 171-193.

Brinster, R. L. 1963. A method for in vitro cultivation of mouse ova from two-cell to blastocyst. Exp. Cell Res. 32, 205-208.

Casillas, E. R. and D. D. Hoskins. 1971. Adenyl cyclase activity and cyclic 3', 5' -AMP content of ejaculated monkey spermatozoa. Archs. Biochem. Biophys. 147, 148-155.

Cline, E. M., K. G. Gould and C. W. Foley. 1972. Regulation of ovulation, recovery of mature ova of the squirrel monkey (Saimiri sciureus). Fed. Proc. 31, 360.

Cornett, L. E. and S. Meizel. 1978. Stimulation of in vitro activation and the acrosome reaction of hamster spermatozoa by catecholamines. Proc. Nat. Acad. Sci. USA. 75, 4954-4958.

Cornett, L. E., B. D. Bavister and S. Meizel. 1979. Adrenergic stimulation of fertilizing ability in hamster spermatozoa. Biol. Reprod. 20, 925-929.

Davidson, E., V. G. Allfrey and A. E. Mirsky. 1964. On the RNA synthesized during the lampbrush phase of the amphibian oogenesis. Proc. Nat. Acad. Sci. USA. 52, 501-508.

Davis, D. L. and B. N. Day. 1978. Cleavage and blastocyst formation by pig eggs in vitro. J. Anim. Sci. 46, 1043-1053.

Delgado, N. M., L. Huacuja, R. Pancardo, H. Merchant and A. Rosado. 1976. Changes in the protein conformation of human spermatozoal membranes after treatment with cyclic adenosine 3': 5' -monophosphate and human follicular fluid. Fertil.Steril. 27, 413-420.

DeTurner, E. A., N. J. Aparicio, D. Turner and L. Schwarzstein. 1978. Effect of two phosphodiesterase inhibitors, cyclic adenosine 3': 5' -monophosphate, and a -blocking agent on human sperm motility. Fertil.Steril. 29, 328-331.

Dukelow, W. R. 1970. Inducation and timing of single and multiple ovulations in the squirrel monkey (Saimiri sciureus). J. Reprod. Fertil. 22, 303-309.

Dukelow, W. R., S. J. Jarosz, D. A. Jewett and R. M. Harrison. 1971. Laparoscopic examination of the ovaries in goats and primates. Lab. Anim. Sci. 21, 594-597.

Dukelow, W. R. and T. J. Kuehl. 1975. La Fecondation; Colloque de la Societe Nationale pour L'Etude de la Sterilite et de la Fecondite, pp 67-80.

Dukelow, W. R. and S. Ariga. 1976. Laparoscopic techniques for biomedical research. J. Med. Primatol. 5, 82-99.

Dukelow, W. R. 1981. The squirrel monkey. In: J. Hearn (ed.) Reproduction and New World Primates. MTR Press, London (in press).

Edwards, R. G. 1962. Meiosis in ovarian oocytes of adult mammals. Nature (London) 196, 446-450.

Edwards, R. G., R. P. Donahue, T. A. Baramki and H. W. Jones. 1966. Preliminary attempts to fertilize human oocytes matured in vitro. Am. J. Obstet. Gynecol. 96, 192-200.

Edwards, R. G. 1965a. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. Nature (London) 208, 349-351.

Edwards, R. G. 1965b. Maturation in vitro of human ovarian oocytes. Lancet 2, 926.

Edwards, R. G., B. D. Bavister and P. C. Steptoe. 1969. Early stages of fertilization in vitro of human oocytes matured in vitro. Nature (London) 221, 632-635.

Edwards, R. G. and R. E. Fowler. 1970. Human embryos in the laboratory. Scientific American 223, 4454.

Edwards, R. G. 1973a. Physiological aspects of human ovulation, fertilization and cleavage. J. Reprod. Fertil., Suppl. 18, 87-101.

Edwards, R. G. 1973b. Studies on human conception. Am. J. Obstet. Gynecol. 117, 587-601.

Edwards, R. G. and P. C. Steptoe. 1975. Physiological aspects of human embryo transfer. In: S. J. Bahrman and R. W. Kistner (eds.) Progress in Infertility. Little, Brown and Company, Boston.

Fleming, A. D. and R. Yanagimachi. 1980. Superovulation and superpregnanyy in the golden hamster. Develop., Growth and Differ. 22, 103-112.

Fraser, L. 1979. Accelerated mouse sperm penetration in vitro in the presence of caffeine. J. Reprod. Fertil. 57, 377-384.

Fridhandler, L., W. B. Wastila and W. M. Palmer. 1967. The role of glucose in metabolism of the developing mammalian preimplantation conceptus. Fertil. Steril. 18, 819-830.

Fukui, Y. and Y. Sakuma. 1980. Maturation of bovine oocytes cultured in vitro: Relation to ovarian activity, follicular size and the presence or absence of cumulus cells. Biol. Reprod. 22, 669-673.

Garbers, D. L., N. L. First, J. J. Sullivan and H. A. Lardy. 1971a. Stimulation and maintenance of ejaculated bovine spermatozoan respiration and motility by caffeine. Biol. Reprod. 5, 336-339.

Garbers, D. L., W. D. Lust, N. L. First and H. A. Lardy. 1971b. Effects of phosphodiesterase inhibitors and cyclic nucleotides on sperm respiration and motility. Biochemistry, N.Y. 10, 1825-1831.

Goren, E. N. and O. M. Rosen. 1972. Inhibition of a cyclic nucleotide phosphodiesterase from beef heart by catecholamines and related compounds. Molec. Pharm. 8, 380-384.

Gould, K. G., E. M. Cline and W. L. Williams. 1973. Observations on the induction of ovulation and fertilization in vitro in the squirrel monkey (Saimiri sciureus). Fertil.Steril. 24, 260-268.

Gwatkin, R. B. L. and D. F. Andersen. 1969. Capacitation of hamster spermatozoa by bovine follicular fluid. Nature, London 224, 111.

Hardman, J. G., J. L. Hammer, R. T. Hoos and E. W. Sutherland. 1971. Adenyl cyclase, phosphodoesterase and cyclic AMP of human sperm and seminal plasma. Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 30, 1251.

Hayashi, M. 1963. Fertilization in vitro using human ova. Proc. VII Conf. Inter. Planned Parenthood Fed. Singapore, pp 505-510.

Hendrickx, A. G. and D. C. Kraemer. 1968. Preimplantation stages of baboon embryos (Papio sp.). Anat. Rec. 162, 111-120.

Hillënsjo, T., S. H. Pomerantz, A. Schwartz-Kripner, L. D. Anderson and C. P. Channing. 1980. Inhibition of cumulus cell progesterone secretion by low molecular weight fractions of porcine follicular fluid which also inhibit oocyte maturation. Endocrinology 106, 584-591.

Hoskins, D. D. 1973. Adenine nucleotide mediation of fructolysis and motility in bovine epididymal spermatozoa. J. Biol. Chem. 248, 1135-1180.

Hsu, Y. C., J. Basker, L. C. Stevens and J. E. Rash. 1974. Development in vitro of mouse embryos from the two-cell egg stage to the early somite stage. J. Embryol. Exp. Morph. 31, 235-245.

Hsu, Y. C. 1980. Embryo growth and differentiation factors in embryonic sera of mammals. Develop. Biol. 76, 465-474.

Jagiello, G., J. Karnicki and R. J. Ryan. 1968. Superovulation with pituitary gonadotrophins method for obtaining meiotic metaphase figures in human ova. Lancet 1, 178.

Jagiello, G., J. Graffeo, M. Ducayen and R. Prosser. 1977. Further studies of inhibitors of in vitro mammalian oocyte maturation. Fertil. Steril. 28, 476-481.

Johnson, M. P., R. M. Harrison and W. R. Dukelow. 1972. Studies on oviductal fluid and in vitro fertilization in rabbits and nonhuman primates. Fed. Proc. 31, 369.

Kane, M. T. 1979. Fatty acids as energy sources for culture of one-cell rabbit ova to viable morulae. Biol. Reprod. 20, 323-332.

Kaukel, E., K. Mundhenk and H. Hilz. 1972. N^6-monobutyryl adenosine 3' : 5' monophosphate as the biologically active derivitive of dibutyryl adenosine 3' : 5' monophosphate in Hala 53 cells. Eur. J. Biochem. 27, 197-200.

Kennedy, J. F. and R. P. Donahue. 1969. Human oocytes:Maturation in chemically defined media. Science 164, 1292-1293.

Kennedy, J. F. 1970. Maturation and fertilization of human oocytes in vitro. In: J. D. Biggers and A. W. Schultz (eds.) Oogenesis. University Park Press, Baltimore, Maryland.

Kraemer, D. C. 1972. Personal communication (as cited by Kuehl), 1976.

Kraemer, D. C., B. L. Flow, M. D. Schriver, G. M. Kinney and J. W. Pennycook. 1979. Embryo transfer in the nonhuman primate, feline and canine. Theriogenology 11, 51-62.

Kuehl, T. J. 1974. In vitro fertilization in Saimiri sciureus. M.S. Thesis, Michigan State University, E. Lansing, Michigan.

Kuehl, T. J. and W. R. Dukelow. 1975a. Fertilization in vitro of Saimiri sciureus follicular oocytes. J. Med. Primatology 4, 209-216.

Kuehl, T. J. and W. R. Dukelow. 1975b. Ovulation induction during the anovulatory season in Saimiri sciureus. J. Med. Primatol. 4, 23-31.

Kuehl, T. J. 1976. Variables involved in gamete recovery, in vitro fertilization, embryo culture and transfer in the squirrel monkey

(Saimiri sciureus). Ph.D. Thesis, Michigan State University, E. Lansing, Michigan.

Kuehl, T. J. and W. R. Dukelow. 1977. The development of squirrel monkey oocytes following transfer to the rabbit oviduct. Proc. Midwest Reg. Develop. Biol. Conf.

Kuehl, T. J. and W. R. Dukelow. 1979. Maturation and in vitro fertilization of follicular oocytes of the squirrel monkey (Saimiri sciureus). Biol. Reprod. 21, 545-556.

Lewis, W. H. and C. G. Hartman. 1933. Early cleavage stages of the egg of the monkey (Macaca rhesus). Contr. Embryol. Carneg. Instn. 24, 187-201.

Lewis, W. H. and C. G. Hartman. 1941. Tubal ova of the rhesus monkey. Contr. Embryol. Carneg. Instn. 29, 7-14.

Lopata, A., I. W. H. Johnston, J. F. Leeton, D. Muchnicki, J. McTalbot, and C. Wood. 1974. Collection of human oocytes at laparoscopy and laparotomy. Fertil.Steril. 25, 1030-1038.

Lopata, A., R. McMaster, J. C. McBain and W. I. H. Johnston 1978. In vitro fertilization of preovulatory human eggs. J. Reprod. Fert. 52, 339-342.

Lopata, A., J. B. Brown, J. F. Leeton, J. McTalbot and C. Wood. 1978. In vitro fertilization of preovulatory oocytes and embryo transfer in infertile patients treated with clomiphene and human chorionic gonadotropin. Fertil.Steril. 30, 27-35.

Lopata, A., I. W. H. Johnston, I. J. Hoult and A. Z. Speirs. 1980. Pregnancy following intrauterine implantation of an embryo obtained by in vitro fertilization of a preovulatory egg. Fertil.Steril. 33, 117-120.

Lui, C. W., L. E. Cornett and S. Meizel. 1977. Identification of the bovine follicular fluid protein involved in the in vitro induction of the hamster sperm acrosome reaction. Biol. Reprod. 17, 34-41.

Makler, A., E. Makler, J. Itzkovitz and J. M. Brandes. 1980. Factors affecting sperm motility. IV. Incubation of human semen with caffeine, kallikrein, and other metabolically active compounds. Fertil.Steril. 33, 624-630.

Meizel, S., C. W. Lui, P. K. Working and R. J. Mrsny. 1980. Taurine and hypotaurine: Their effects on motility, capacitation and the acrosome reaction of hamster sperm in vitro and their presence in sperm and reproductive tract fluids and their presence in sperm and reproductive tract fluids of several mammals. Develop., Growth and Differ. 22, 483-494.

Meizel, S. and P. K. Working. 1980. Further evidence suggesting the hormonal stimulation of hamster sperm acrosome reactions by catecholamines in vitro. Biol. Reprod. 21, 211-216.

Mizoguchi, H. and W. R. Dukelow. 1980. Gradual fixation method for chromosomal studies of squirrel monkey oocytes after gonadotropin treatment. J. Med. Primatol. (in press).

Moor, R. M. and G. M. Warnes. 1979. Regulation of meiosis in mammalian oocytes. British Med. Bull. 35, 99-103.

Moore, H. D. M. and J. M. Bedford. 1978. An in vitro analysis of factors influencing the fertilization of hamster eggs. Biol. Reprod. 19, 879-885.

Morton, B. and T. S. K. Chang. 1973. The effect of fluid from the cauda epididymis, serum components and caffeine upon the survival of diluted epididymal hamster spermatozoa. J. Reprod. Fert. 35, 255-263.

Mrsny, R. J. and S. Meizel. 1980. Evidence suggesting a role for cyclic nucleotides in acrosome reactions of hamster sperm in vitro. J. Exp. Zool. 211, 153-157.

New, D. A. T. 1967. Development of explanted rat embryos in circulating medium. J. Embryol. Exp. Morph. 17, 513.

New, D. A. T.,P. T. Coppola and S. Terry. 1973. Culture of explanted rat embryos in rotating tubes. J. Reprod. Fert. 35, 135-138.

Niwa, K., H. Imai, C. I. Kim and A. Iritani. 1980. Fertilization in vitro of hamster and mouse eggs in a chemically defined medium. J. Reprod. Fert. 58, 109-114.

Onuma, H., R. R. Maurer and R. H. Foote. 1968. In vitro culture of rabbit ova from early cleavage stages to the blastocyst stage. J. Reprod. Fert. 16, 491-493.

Pienkowski, M. M. 1980. Modulation of cell proliferation in cultured postimplantation embryos by cyclic AMP. Biol. Reprod. 22, 582-586.

Pincus, G. and B. Saunders. 1939. The comparative behavior of mammalian eggs in vivo and in vitro. Vi. The maturation of human ovarian ova. Anat. Rec. 75, 537-544.

Pope, C. E., V. Z. Pope and L. R. Beck. 1980. Nonsurgical recovery and in vitro culture of baboon embryos. Arch. Androl. 5, 99-105.

Posternak, Th., E. W. Sutherland and W. R. Henion. 1962. Derivatives of cyclic 3', 5' adenosine monophosphate. Biochem. Biophys. Acta 65, 558-560.

Quinn, P. and R. G. Wales. 1973. The relationships between the ATP content of preimplantation mouse embryos and their development in vitro during culture. J. Reprod. Fert. 35, 301-309.

Renard, J.-P, A. Phillippon and Y. Menezo. 1980. In vitro uptake of glucose by bovine blastocysts. J. Reprod. Fert. 58, 161-164.

Reyes, A., B. Goicorchea and A. Rosado. 1978. Calcium ion requirement for rabbit spermatozoal capacitation and enhancement of fertilizing ability by ionophore A23187 and cyclic adenosine 3':5' -monophosphate. Fertil.Steril. 29, 451-455.

Rock, J. and M. F. Menkin. 1944. In vitro fertilization and cleavage of human ovarian eggs. Science 100, 105-107.

Rogers, B. J. and L. Garcia. 1979. Effect of cAMP on acrosome reaction and fertilization. Biol. Reprod. 21, 365-372.

Rosado, A., J. J. Hicks, A. Reyes and I. Blanco. 1974. Capacitation in vitro of rabbit spermatozoa with cyclic adenosine monophosphate and human follicular fluid. Fertil.Steril. 25, 821-824.

Rosenblum, L. A. and R. W. Cooper (eds.) 1968. The squirrel monkey. Academic Press, New York, London.

Santos-Sacchi, J., M. Gordon and W. L. Williams. 1980. Potentiation of the cGMP-induced guinea pig acrosome reaction by zinc. J. Exp. Zool. 213, 289-291.

Schoenfeld, C., R. D. Amelar and L. Dubin. 1975. Stimulation of ejaculated human spermatozoa by caffeine. Fertil.Steril. 26, 158-161.

Seitz, H. M., G. Rocha, B. G. Brackett and L. Mastroianni. 1971. Cleavage of human ova in vitro. Fertil.Steril. 22, 255-262.

Shea, B. F., R. D. Baker and J. P. A. Latour. 1975. Human follicular oocytes and their maturation in vitro. Fertil.Steril. 26, 1075-1082.

Smith, D. M., C. H. Conaway and W. T. Kerber. 1978. Influences of season and age on maturation in vitro of rhesus monkey oocytes. J. Reprod. Fert. 54, 91-95.

Soupart, P. and L. L. Morgenstern. 1973. Human sperm capacitation and in vitro fertilization. Fertil.Steril. 24, 462-477.

Soupart, P. and P. A. Strong. 1974. Ultrastructural observations on human oocytes fertilized in vitro. Fertil.Steril. 25, 11-44.

Soupart, P. and P. A. Strong. 1975. Ultrastructural observations on polyspermic penetration of zona pellucida-free human oocytes inseminated in vitro. Fertil.Steril. 26, 523-537.

Stambaugh, R. and L. Mastroianni, Jr. 1980. Stimulation of rhesus monkey (Macaca mulatta) proacrosin activation by oviduct fluid. J. Reprod. Fert. 59, 479-484.

Steptoe, P. C. and R. G. Edwards. 1970. Laparoscopic recovery of preovulatory human oocytes after priming of ovaries with gonadotrophins. Lancet 1, 683.

Steptoe, P. C. and R. G. Edwards. 1976. Reimplantation of a human embryo with subsequent tubal pregnancy. Lancet 1, 880.

Suzuki, S. and L. Mastroianni, Jr. 1966. Maturation of monkey ovarian follicular oocytes in vitro. Am. J. Obstet. Gynecol. 96. 723-731.

Thibault, C., M. Gerard and Y. Menezo. 1976. Nuclear and cytoplasmic aspects of mammalian oocyte maturation in vitro in relation to follicle size and fertilization. In: Sperm action. Prog. Reprod. Biol. i, 233-240.

Thibault, C. 1977. Are follicular maturation and oocyte maturation independent processes? Hammond Memorial Lecture. J. Reprod. Fert. 51, 1-15.

Thompson, R. S. and L. Zamboni. 1975. Anomalous patterns of mammalian oocyte maturation and fertilization. Am. J. Anat. 142, 233-263.

Toyoda, Y. and M. C. Chang. 1974. Capacitation of epididymal spermatozoa in a medium with high K/Na ratio and cyclic AMP for the fertilization of rat eggs in vitro. J. Reprod. Fert. 36, 125-134.

Tsafiri, A. and C. P. Channing. 1975. An inhibitory influence of granulosa cells and follicular fluid upon procine oocyte meiosis in vitro. Endocrinology 96, 922.

Upchurch, B. J. and B. E. Morton. 1972. ATP levels in hamster spermatozoa during capacitation in vitro. Abstract no. 92. Society for the Study of Reproduction, 5th Meeting, E. Lansing, Michigan.

Willingham, M. C. and I. Pastan. 1975. Cyclic AMP and cell morphology in cultured fibroblasts. Effects on cell shape, microfilament, and microtubule distribution, and orientation to substratum. J. Cell Biol. 67, 146-159.

Wincek, T. J., R. F. Parrish and K. L. Polakoski. 1979. Fertilization: a uterine glycosaminoglycon stimulates the conversion of sperm proacrosin to acrosin. Science, N.Y. 203, 553-554.

Yanagimachi, R. and M. C. Chang. 1963. Fertilization of hamster eggs in vitro. Nature, London 200, 281-282.

Yanagimachi, R. and M. C. Chang. 1964. In vitro fertilization of hamster ova. J. Exp. Zool. 156, 361-376.

Yanagimachi, R. 1969. In vitro acrosome reaction and capacitation of golden hamster sperm by bovine follicular fluid and its fractions. J. Exp. Zool. 170, 269-280.

Yanagimachi, R. 1970a. The movement of golden hamster spermatozoa before and after capacitation. J. Reprod. Fert. 23, 193-196.

Yanagimachi, R. 1970b. In vitro capacitation of golden hamster spermatozoa by homologous and heterologous blood sera. Biol. Reprod. 3, 147-153.

Zamboni, L., R. S. Thompson and D. M. Smith. 1972. Fine morphology of human oocyte maturation in vitro. Biol. Reprod. 7, 425-457.

APPENDIX

Publications by the Author

Full Papers

1) Sex- and age-related mouse toxicity and disposition of the amino acid antitumor agent, Acivicin. J. P. McGovern, G. L. Neil, P. C. S. Chan and J. C. Stewart. J. Pharmacol. Exp. Therap. 216, 433-440, 1981.

2) Alternative methods of fertilization for reproductive toxicology. W. R. Dukelow, P. J. Hirst, T. Asakawa, F. J. DeMayo and P. J. Chan. Proceedings of the 8th European Teratology Conference. 1981. (in press).

3) Cyclic nucleotide effects on preimplantation development of the squirrel monkey (Saimiri sciureus) embryo fertilized in vitro. P. J. Chan and W. R. Dukelow. Exptl. Zool. 1981 (Submitted).

4) Time sequence of in vitro maturation and chromosomal normality in metaphase I and metaphase II of squirrel monkey oocytes. T. Asakawa, P. J. Chan and W. R. Dukelow. Biol. Reprod. 1981 (Submitted).

Abstracts

1) Nonhuman primate in vitro fertilization and development: effects of culture additives. P. Chan, T. Asakawa and W. R. Dukelow. Proc. 31st Annual Mtg. Amer. Assoc. for Lab. Anim. Sci. 1980. Indianapolis, Indiana.

2) Nonsurgical (laparoscopic) ovum and embryo recovery techniques in the squirrel monkey and mink. F. J. DeMayo, P. Chan and W. R. Dukelow. Proc. 31st Annual Meeting of Amer. Assoc. for Lab. Anim. Sci. 1980. Indianapolis, Indiana

3) Alternatives to natural fertilization in primates. W. R. Dukelow, p. J. Chan, F. J. DeMayo and M. T. Ridha. American Society of Primatologists. 1980. Winston-Salem, No. Carolina.

4) Cyclic nucleotide involvement in capacitation and the acrosome reaction as assessed in a primate in vitro fertilization system. P. J. Chan, T. Asakawa and W. R. Dukelow. Proc. Society for the Study of Reproduction. 1981. Corvallis, Oregon.

5) In vitro fertilization of nonhuman primate ova. W. R. Dukelow and P. J. Chan, Proc. American Society of Primatologists. 1981. San Antonio, Texas.

VITA

Name:	Philip Chan Chee Sem
Born:	May 11, 1956
Education:	St. Michael's Institution Ipoh Malaysia
	Kalamazoo College Kalamazoo, Michigan
	Michigan State University East Lansing, Michigan
Degrees Received:	Bachelor of Arts (cum laude) Kalamazoo College (1979)
Honors:	Kalamazoo College Honors (1976-1979)