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VARIOUS ASPECTS OF IN VITRO FERTILIZATION AND CULTURE
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VARIOUS ASPECTS OF *IN VITRO* FERTILIZATION AND CULTURE OF PREIMPLANATION SQUIRREL MONKEY EMBRYOS

By

Donna Lynn Pierce

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ABSTRACT

VARIOUS ASPECTS OF *IN VITRO* FERTILIZATION AND CULTURE OF PREIMPLANTATION SQUIRREL MONKEY EMBRYOS

By

Donna Lynn Pierce

The use of nonhuman primates as models for *in vitro* fertilization and preimplantation development is significant for examining the events surrounding primate reproductive physiology. An *in vitro* fertilization system utilizing squirrel monkeys was used to compare follicle-stimulating hormone, clomiphene citrate and prostaglandin E1 as follicular induction regimens, analyze culture medium characteristics, and examine the physiological phenomenon of polyspermy. Induction of follicular growth was poor with 2 and 4 mg of clomiphene citrate when compared to the control group. When prostaglandin E1 was administered, larger numbers of mature oocytes were recovered at laparoscopy. There was no difference in fertilization rate between the treatment and control groups. There was a strong inverse relationship (r^2=-0.98) between calcium level and *in vitro* maturation. Fertilization rate and calcium concentration in the medium

were closely related (r^2 =0.90). There was no relationship between sperm concentration and the incidence of polyspermy. These findings demonstrate that the squirrel monkey is a useful system for examining various aspects of *in vitro* fertilization and early embryonic development requirements in nonhuman primates.

You who were darkness warmed my flesh
where out of darkness rose the seed.
Then all a world I made in me;
all the world you hear and see
hung upon my dreaming blood.

There moved the multitudinous stars,
and coloured birds and fishes moved.
There swam the sliding continents.
All time lay rolled in me, and sense,
and love that knew not its beloved.

O node and focus of the world;
I hold you deep within that well
you shall escape and not escape-
that mirrors still your sleeping shape;
that nurtures still your crescent cell.

I wither and you break from me;
yet though you dance in living light
I am the earth, I am the root,
I am the stem that fed the fruit,
the link that joins you to the night.

Judith Wright

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INTRODUCTION

In vitro fertilization of mammalian oocytes was initially described in pioneering studies by Chang (1951). Interest in this technique was aroused further with the announcement by Edwards et al. (1969) of successful *in vitro* fertilization in the human followed by the birth of Louise Brown in 1978. *In vitro* fertilization and embryo transfer have now become a therapeutic technique for human infertility. However, ethical and moral restrictions prevent testing and research development of *in vitro* fertilization systems in the human so nonhuman primates are very valuable for this purpose.

A system has been developed that allows consistent follicular induction, oocyte collection, semen collection and *in vitro* fertilization to produce embryos for the study of basic embryological development in the squirrel monkey. The achievement of *in vitro* fertilization enables the researcher to monitor metabolic activity and biochemical changes, as well as the chromosomal and temporal normality of developing embryos. The temporal development through cleavage stages for the squirrel monkey as compared to

TABLE 1

Comparative Rates of Primate Preimplantation Development

	Hours after fertilization				
	Squirrel Monkey	Rhesus Macaque	Cynomolgus Macaque	Baboon	Human
Two-Polar Bodies	6-22			6-24	12
2-Cell	16-40	24-36	24	24	30-38
4-Cell	45-52	36-48	48	48	38-46
8-Cell	52-72	48-72	48-72	48-72	51-72
16-Cell		72-96		96-120	85-96
Morula				120-148	96-135
Blastocyst	96			96-144	123-147

other nonhuman species and humans is shown in Table 1 (Dukelow, 1983a). It is evident that the stages of development are quite comparable and this lends credibility to the use of the squirrel monkey as a model for studying human in vitro fertilization.

The required growth factors that are necessary for progression of embryos to later stages of development have not been fully examined in the squirrel monkey. The addition of dibutyrl cAMP in TC-199 culture medium has increased fertilization rates from 60% to 90% (Chan et al., 1982). There have been no reports of squirrel monkey embryos consistently cleaving and growing in vitro beyond the eight cell stage. Progress in this area would provide insight on the basic physiology of the primate embryo as well as have clinical significance to human in vitro fertilization.

The technique of in vitro fertilization can be used to study basic physiological phenomena such as polyspermy. In rodents, it is known that in vitro fertilization results in a greater incidence of triploidy (caused by polyspermy) than fertilization in vivo. Chromosomal abberations are responsible for a substantial amount of reproductive inefficiency and birth defects. Reduced fertility, sterility, embryonic or fetal death, stillbirths and congenital malformations may all arise from a change in chromosome number. The techniques of ovarian stimulation, ova collection, *in vitro* fertilization and embryo transfer have generated concern regarding risks of inducing abnormalities in the embryo or fetus. In order to understand the risks appropriately, the role of chromosomal abberations must be considered in mammalian reproduction.

In vitro fertilization and embryo transfer in nonhuman primates has been reviewed (Dukelow, 1983a,b,c). The technique has been successfully used in several primate species: the squirrel monkey, the marmoset, the baboon, the rhesus macaque, the cynomolgus macaque and the chimpanzee. Unsuccessful attempts at *in vitro* fertilization have also been made in the Western Lowland Gorilla.

The basic objectives of this research were:

1) To determine some of the required development factors in the squirrel monkey that enable embryos to proceed to further stages of development.

2) To examine the fertilizability of superovulated oocytes in the monkey as a possible source of greater numbers of embryos available for experimental testing.

3) To assess the cause of polyspermy which is present in nearly all *in vitro* fertilization systems (human and animal species) and reduce the incidence of polyspermy.

VARIOUS ASPECTS OF *IN VITRO* FERTILIZATION AND CULTURE OF PREIMPLANTATION SQUIRREL MONKEY EMBRYOS

LITERATURE REVIEW

BASIC REPRODUCTION OF THE SQUIRREL MONKEY

The squirrel monkey (*Saimiri sciureus*) is used widely in research due to its small size, ease of handling and basic housing requirements. In addition, a strong background on reproduction exists for this species. The cycle length varies from 7-12 days with an average of 9 days having been determined by vaginal cell cornification cycles (Rosenblum et al., 1967), behavioral characteristics (Richter, 1976; Latta et al., 1967; Jarosz et al., 1977) and radioimmunoassay of circulating steroids (Wolf et al., 1977; Wilson, 1977; Ghosh et al., 1982).

Reproductive seasonality has been described in the squirrel monkey and is manifested by behavioral changes, dimorphic appearence of the adults ("fatted male syndrome") (Dumond and Hutchinson, 1967) and hormonal variations (Diamond et al., 1984). Squirrel monkeys mate from July to September with births occurring from January to March in their natural habitat. The mating season shifts to January through March when moved to the northern hemisphere with births occurring in the summer months (Dumond, 1968).

Adaptation to captivity must be taken into consideration when establishing a breeding colony. Some animals will adjust after nine months of captivity (Harrison and Dukelow, 1973), while others will require as long as three breeding seasons to adapt (Lorenz et al., 1973).

The seasonal effect of lowered ovulation is evident in the female response to ovulation induction regimens in the laboratory (Harrison and Dukelow, 1973). Follicular growth and ovulation can be induced during the anovulatory months by increasing the dose of follicle stimulating hormone (FSH) from 1 to 2 mg per day or extending the 1 mg dose to five days instead of four days (Kuehl and Dukelow, 1975a).

FOLLICULAR INDUCTION-NONHUMAN PRIMATES

Induction of ovulation in some species of old world monkeys was initially reported by injecting extracts of monkey pituitaries and human menopausal gonadotropin (HMG) (Van Wagenen and Simpson, 1957; Simpson and Van Wagenen, 1962). The first experiments in new world primates were described by Bennett (1967) who used injections of pregnant mares serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) to induce follicular growth and ovulation and furthermore, superovulation was achieved with higher doses of PMSG. However, it has been found that repeated administration of PMSG to an animal would result in a refractoriness to the PMSG probably due to its antigenicity (Gould et al., 1973; Ozasa and Gould, 1987). Since captive squirrel monkeys used in biomedical research are subjected to several follicular induction regimens, studies were undertaken to induce single or double ovulations in the squirrel monkey (Dukelow, 1970). Monkeys were pretreated with 5 mg of progesterone for 5 days to mimic the luteal phase of the cycle. The animals were then given various treatments of follicle stimulating hormone, human menopausal gonadotropin and pregnant mares serum. The optimal regimen consisted of four days of 1 mg of FSH followed by a single injection of 250-500 IU of HCG on the last day. Oocytes produced from follicular induction regimens are capable of being fertilized both in vivo (Jarosz et al., 1977) and in vitro (Kuehl and Dukelow, 1975b, 1979; Chan et al., 1982).

Recently, induction of follicular growth in the squirrel monkey has been achieved using human urinary follicle stimulating hormone (hFSH) (Yano and Gould, 1985). The authors reported hFSH to be as effective as HMG for inducing follicle development and was superior to both HMG and pure follicle stimulating hormone (pFSH) at lower dose rates.

The involvement of prostaglandins either directly or indirectly in mammalian ovulation has been shown in the rhesus monkey (Batta and Brackett, 1974; Wallach et al., 1975; Batta et al., 1978). A consistent method for producing superovulation in the rhesus monkey using a combination of gonadotropins and prostaglandin E1 was reported by Batta et al. (1978). More recently, controlled ovulation has been achieved in the marmoset with human chorionic gonadotropin and prostaglandins (Hodges et al., 1987). The use of gonadotropins and prostaglandins may be important for primate experiments involving oocyte collection, oocyte maturation and synchronization of recipients in embryo transfer.

FOLLICULAR INDUCTION-HUMANS

There are several methods used in human *in vitro* fertilization for ovarian stimulation. These include: 1) clomiphene citrate with spontaneous luteinizing hormone surge or human chorionic gonadotropin injection; 2) human menopausal gonadotropin and human chorionic gonadotropin; 3) sequential treatment with clomiphene citrate, human menopausal gonadotropin and human chorionic gonadotropin; 4) pure follicle stimulating hormone and human chorionic gonadotropin or pure follicle stimulating hormone alone and 5) pulsatile gonadotropin releasing hormone (GnRH).

Holman and Hammond (1988) have recently reviewed the subject of ovulation induction with clomiphene citrate. Treatment regimens for ovarian stimulation using clomiphene citrate generally consist of a 100-150 mg dose given daily starting on the third to fifth day after the start of menses (Trounson et al., 1981; Templeton et al., 1986). Ultrasound and blood sampling for estradiol levels began on the eighth or ninth day of the menstrual cycle. Laparoscopy was performed 26-36 hours after spontaneous luteinizing hormone surge or HCG injection. Estradiol levels were usually above 500 pg/ml and follicle diameter greater than 17 mm. Trounson et al. (1981) recovered 41 mature oocytes from 30 patients. Many _in vitro_ fertilization programs have abandoned using clomiphene citrate alone since the number of oocytes recovered and pregnancy rates were lower than with other methods of stimulation.

Garcia et al. (1983) chose to stimulate follicular growth with human menopausal gonadotropin and human chorionic gonadotropin to induce follicular maturation because they were considered "physiologic hormones" and provided a more predictable ovarian response. Human menopausal gonadotropin was administered intramuscularly daily starting on the third day with estradiol levels and follicle size monitored. When estradiol concentrations rose above 300 pg/ml and the follicle diameter exceeded 14 mm, human menopausal gonadotropin was discontinued and HCG administered 50 hours later. An average of 4.7 ova were retrieved per laparoscopy with 23% of the transfers resulting in a pregnancy.

Combination therapy is considered to be preferable to standard menotropin therapy since it decreases the risks of hyperstimulation and quantity of HMG needed (Kemman and Jones, 1983). Sequential treatment regimens have been used successfully by Lopata (1983) recovering approximately five oocytes per laparoscopy.

The use of human urinary follicle stimulating hormone (Metrodin^{R}) and human menopausal gonadotropin for inducing multiple follicular growth and maturation has been discussed by Muasher et al. (1985). They retrieved an average of three preovulatory oocytes per laparoscopy and reported a pregnancy rate of 27% per transfer cycle.

More recently, another study compared pure follicle stimulating hormone to human menopausal gonadotropin (Russell et al. 1986). The fertilization rates were not significantly different, but the cleavage and pregnancy rates were superior with the use of pure FSH. The authors attributed the difference to pure FSH being more physiologic in directing follicular steroidogenesis, thus providing an optimal environment for oocyte development.

The area of ovulation induction with gonadotropin-releasing hormone has recently been reviewed (Zacur, 1985; Lunenfeld et al., 1988) and is a promising new area of fertility regulation.

IN VITRO FERTILIZATION-NONHUMAN PRIMATES

The area of *in vitro* fertilization and embryo transfer (IVF-ET) in nonhuman primates has been reviewed (Dukelow, 1983b,c). *In vitro* fertilization in the squirrel monkey was first announced in 1972 (Cline et al.; Johnson et al.) with the first full publication of the former workers appearing in 1973 (Gould et al.). These authors reported recovery of 22 mature oocytes, 11 of which had sperm in the perivitelline space, extrusion of the second polar body or formation of pronuclei. Later studies achieved a 40% fertilization rate (Kuehl and Dukelow, 1975b). More recently, the effect of varying quantities of cumulus cells on squirrel monkey *in vitro* fertilization was studied (Chan et al., 1982). When cumulus cells were not present, oocyte maturation was reduced, but when oocytes were covered with at least one quarter cumulus cells, a 70% fertilization rate was observed. It was also shown that if ova collected 15-16 hours after HCG injection were allowed to incubate an additional 21 hours before sperm introduction, levels of fertilization were higher. In addition, supplementation with 1 micromole of dibutyrl cAMP to the culture medium increased fertilization from 60% to 90%. This effect probably resulted from a stimulation of sperm capacitation, acrosome reaction and whiplash motility which are all required for oocyte penetration.

Successful *in vitro* fertilization has been acomplished with cynomolgus monkey (Kreitman et al., 1982; Balmaceda et al., 1984) oocytes after incubation with homologous sperm. Bavister et al. (1983) used a superovulation regimen which consisted of 12 days PMSG beginning on day 3-5 in the rhesus monkey. Fertilization *in vitro* was achieved at a rate of 43% with 79% of fertilized oocytes undergoing at least one cleavage.

Kuehl (1983) and Irsigler et al. (1984) described *in vitro* fertilization with baboon follicular oocytes with the former researcher reporting 61% fertilization. Clayton and Kuehl (1984) were the first to document a live birth of a baboon infant resulting from *in vitro* fertilization and embryo transfer. Subsequently, a healthy rhesus offspring was born from IVF-ET (Bavister et al., 1984). These experiments are crucial since conclusive evidence can be obtained from the birth of a normal infant after embryo transfer that the procedures are not harmful to the offspring and provide an experimental model for studying fertilization and embryogenesis in primates.

The first documentation of *in vitro* fertilization in the chimpanzee was reported by Gould (1983). Clomiphene citrate was administered for five days starting on the second day of perineal swelling with HCG given 72 hours after the last dose of clomiphene citrate. A total of 66 oocytes were collected in 50 attempts with 52% of those ova mature resulting in a 42% fertilization rate.

IN VITRO FERTILIZATION-HUMANS

The first attempts at *in vitro* fertilization of human oocytes were reported by Rock and Menken (1944) and Edwards et al. (1966). Successful *in vitro* fertilization of seven mature oocytes was documented by Edwards et al. (1969). However, five of the oocytes were polyspermic. Bavister et al. (1969) observed sperm midpiece and tail in 69% of fertilized oocytes 10.5 to 24 hours after insemination. Steptoe and Edwards (1970) utilized laparoscopy for oocyte recovery and were able to culture human embryos to the blastocyst stage *in vitro* (Steptoe et al., 1971).

Soupart and Morgenstern (1973) investigated the effect of follicle stimulating hormone, luteinizing hormone and human chorionic gonadotropin on sperm penetration *in vitro*. The fertilization rate was 12.5% in the presence of these hormones compared to 0% for controls. The authors concluded that the need for capacitation of human sperm was enhanced by the hormones and the hormone action was mediated by the follicle cells.

The first baby born from *in vitro* fertilization was announced in 1978 by Steptoe and Edwards. Their techniques were not published until late in 1980. The Australians were the next to repeat the success of the British (Lopata et al., 1980) and certain procedural modifications led to a significant increase in the success rate (Trounson et al., 1981). Two human *in vitro* fetilization groups have reviewed their programs over a three to four year period (Edwards and Steptoe, 1983; Jones et al., 1984) and are reporting pregnancy rates of 30% and 25%, respectively.

A variation of *in vitro* fertilization called gamete intrafallopian transfer (GIFT) has been proposed as a new infertility treatment (Asch et al., 1984). It is an attractive alternative to *in vitro* fertilization since initial reports cite a 29% to 40% pregnancy rate and has been successful with some cases of idiopathic infertility (Asch et al., 1986a). These patients possess anatomically intact fallopian tubes, theoretically are capable of normal gamete transport and therefore, present an intrafollopian environment more conducive to normal fertilization and embryo development. The results of the latest trials have been summarized (Asch et al., 1986b; Quigley et al., 1987) and 29% and 37% fertilization rates, respectively have been reported.

CULTURE CONDITIONS

Mammalian fertilization occurs in a well-controlled and protected environment within the oviduct. Due to the difficulty of studying ferilization *in vivo*, the technique of *in vitro* fertilization has been utilized to examine and attempt to understand this critical stage of embryonic development. However, *in vitro* fertilization procedures are not always well-defined and success is less than expected in many mammalian species. This situation could be improved by analyzing various components of the *in vitro* fertilization system and utilizing optimum procedures. An optimum culture medium would allow essential changes to occur in spermatozoa (capacitation and acrosome reaction) and support fertilization with subsequent embryo development. *In vitro* fertilization has been accomplished by using a simple technique of placing a micro drop of culture medium into a petri dish, covering it with a layer of mineral oil, equilibrating with 5% CO_2 in air, then adding eggs and sperm and incubating at 37 ^{o}C (Brinster, 1963).

Much of the available information on culture conditions comes from studies involving the mouse, rabbit, hamster and guinea pig (Toyoda et al., 1971; Fraser et al. 1971; Brackett and Oliphant, 1975; Yanagimachi and Chang, 1964; Yanagimachi, 1972). Culture media that have been used successfully for *in vitro* fertilization are modifications of Krebs-Ringer-bicarbonate solution or are based on Tyrodes solution (Hoppe and Pitts, 1973; Bavister and Yanagimachi, 1977). The parameters (ionic composition, osmotic pressure, pH, gas atmosphere and protein source) known to be important in somatic cell function have been studied in these media.

The ionic composition of culture media currently used for *in vitro* fertilization is similar to blood serum from several mammalian species (Altman, 1961). Tervit et al. (1972) attempted to formulate a culture medium analogous to the biochemical composition of sheep oviductal fluid. This medium supported growth of bovine, ovine and caprine embryos to the blastocyst stage. A similar experiment was performed in a human *in vitro* fertilization system with a culture medium formulated on the composition of human tubal fluid (Quinn et al., 1985). They reported that significantly more blastocysts developed in the medium based on human tubal fluid than in modified Tyrodes and attributed this difference to higher potassium levels in the human tubal medium. Similar results were documented in a mouse *in vitro* fertilization system (Roblero and Riffo, 1986) with optimum development at 4.7 mM to 25 mM of potassium.

There has been no sharply defined osmotic pressure optimum for *in vitro* fertilization culture medium in any mammalian species. Hamster and mouse oocytes can be fertilized in medium with osmotic pressures of 177-496 and 250-388mOsm, respectively (Miyamoto and Chang, 1973) with optimal results obtained at 270-402 and 299-305 mOsm, respectively. Culture media are often formulated with an osmotic pressure of 308 mOsm which approximates that of body fluids (Brinster, 1965). Work has shown an optimum osmotic pressure to be lower than accepted physiological levels (Gwatkin and Haidri, 1973; McGaughay, 1977; Bae and Foote, 1980).

The pH of culture medium does seem to be critical. Bavister (1969) reported the optimum pH in the hamster system to be between 7.6 and 7.8 for optimal fertilization. However, these culture conditions were undefined. Several pH buffers used in the hamster system have been evaluated (Bavister, 1982) and it was found that the bicarbonate-CO_2 buffer system yielded the best overall results. HEPES (Good et al., 1966) and TES were adequate pH buffers but anomalies were observed in pronuclei formation. Recently, Bavister (1982) described a well-defined and controlled culture system for *in vitro* fertilization of hamster oocytes with an optimal pH of 7.4 resulting in approximately a 90% fertilization rate.

There have been several reports of successful culture of preimplantation ovine embryos in various media under a 5% CO_2 in air atmosphere (Moor and Cragle, 1971; Peters et al., 1977). Reduction of the oxygen tension from 20 % to 5% was shown to be important for development of one-cell mouse embryos (Whitten, 1971). In contrast, Wright et al. (1976) and Betterbed and Wright (1985) reported no significant difference in sheep embryos cultured under 5% CO_2 in air or 90% N_2, 5% CO_2 and 5% O_2. Carney and Bavister (1987) demonstrated that culturing in a 10% CO_2 environment with TALP medium had a stimulatory effect on in vitro development of hamster embryos probally due to the maintenance of pH. The authors suggest that CO_2 may enhance embryo development through its action as a weak acid in order to maintain the appropriate pH.

Protein is an important substituent of culture medium. Near the time of ovulation, total protein levels in oviductal fluid are 6-20 times lower when compared to blood serum protein concentrations in monkeys and humans (Mastroianni et al., 1969, 1972). The levels of bovine serum albumin (BSA) in commonly used media (3mg/ml) is slightly lower than the physiological concentration. The effect of protein supplementation on one-cell mouse embryos in vitro was examined by Ogawa and Marrs (1987). They reported that the type and timing of protein supplementation of culture medium is significant to embryo growth and that human cord serum may be toxic to embryo survival. Saito et al. (1984) reported that 10% serum was necessary to obtain beneficial conditions for mouse embryo growth and at higher concentrations, did not show any toxic effects or decrease growth rate. The beneficial effects of fetal calf serum on mouse oocytes fertilized *in vitro* were described by Choi et al. (1987). Addition of 10% fetal calf serum to modified Krebs-Ringer-bicarbonate medium improved the incidence of sperm penetration to 80% as compared to medium without fetal calf serum.

In human *in vitro* fertilization, two types of serum, maternal serum and fetal cord serum, are commonly used as 10-20% of the culture medium. Leung et al. (1984) reported that fetal cord serum was a better supplement than maternal serum in culture medium used for human *in vitro* fertilization and embryo development. However, these results could be attributed to the fact that the patients were given clomiphene citrate for ovulation induction which is known to be excreted slowly from the body (Schreiber et al., 1966). Thomson (1968) found clomiphene citrate to have deleterious effects on mouse embryos cultured *in vitro* and suggested that the poorer results could be due the presence of clomiphene citrate in the maternal serum. Ball et al. (1985) examined maternal serum and fetal cord serum in their *in vitro* fertilization system which used human menopausal gonadotropin for ovarian stimulation. They found no difference in fertilization rate, embryo normality or embryo development between fetal cord serum and homologous serum.

POLYSPERMY

The incidence of triploidy caused by polyspermy is known to be greater in *in vitro* fertilization systems as compared to fertilization *in vivo*. The rate of polyspermy has been shown to depend on a range of experimental variables, e.g., sperm concentration, time of preincubation of spermatozoa, age of eggs and the genotype of the gametes (Wolf and Inoue, 1976; Kaleta, 1977; Wolf, 1978). Polyspermy preventing mechanisms in the hamster have been discussed (Barros and Yanagimachi, 1972), and it was suggested that *in vitro* culture delays had an adverse effect or weakened the polyspermy preventing mechanisms at the zona pellucida and vitelline membrane resulting in an abundance of capacitated sperm and multiple entry. Similarly, mouse eggs that were recovered more than two hours prior to ovulation lacked nuclear and cytoplasmic maturation, allowing larger numbers of sperm to penetrate the zona pellucida (Iwamatsu and Chang, 1971). Immediately following oocyte recovery, the cortical granules may still be forming and migrating to the surface of the vitelline membrane (Sathananthan and Trounson, 1982). Thus, supernumerary sperm can enter the oocyte before it is capable of discharging sufficient granules to enable the zona reaction to occur.

Barros et al. (1972) reported that sperm continually penetrated an egg in an *in vitro* culture system over a four hour period and also demonstrated a high correlation between higher incidence of polyspermic eggs with higher pH levels. Sperm concentration (or the ratio of sperm to eggs) could possibly have an effect on the incidence of polyspermy and this question was examined by Siddiquey and Cohen (1982). They found the optimal sperm to egg ratio to be 250-350 and that fertilization was better when lower concentrations of sperm were used and cumulus cells and oviductal fluid were present in the inseminating medium. They concluded that sperm-egg collision rate was the most significant factor in determining fertilization rate. Tsunoda and Chang (1975) also described sperm numbers involved in penetration of mouse eggs *in vitro*.

The effect of estrogen and progesterone both *in vivo* and *in vitro* on the incidence of polyspermy were found to have no effect on mouse eggs (Fraser and Maudlin, 1979). Niemierko and Komai (1976) reported that cytochalasin B weakened the zona reaction in mice and interfered with gamete fusion but did not prevent the block to polyspermy.

The effect of sperm concentration (Wolf et al., 1984) and various ovarian stimulation prototcols have been examined in several human in vitro fertilization programs (Diamond et al., 1985a; Diamond et al.,1985b). Wolf et al. (1984) reported maximum fertilization (80.8%) occurred at a concentration of $2.5x10^4$ motile sperm/ml. The incidence of polyspermy was directly related to sperm concentration increasing from 0% at $1-2.2x10^4$ to 5.5% at $10x10^4$ motile sperm/ml. An inverse relationship between fertilization and sperm concentration was observed with immature oocytes cultured in vitro and inseminated with a maximum fertilization rate of 66.6% at $5x10^4$ motile sperm/ml. Diamond et al. (1985a) examined sperm concentrations and several ovulation induction protocols with respect to polyspermic fertilization. Polyspermy occurred equally in mature and immature oocytes at a rate of 10.2% and a significant difference in polyspermy was observed between stimulating regimens using clomiphene citrate (CC) and those using human menopausal gonadotropin (hMG) or those combining hMG and CC. More recent work has described the incidence of triploidy of in vitro fertilized squirrel monkey oocytes to be 16.7% (Asakawa and Dukelow, 1982). Triploidy is commonly reported in laboratory species in an in vitro fertilization system with similar results in human in vitro fertilization. The exact cause is not known but studies with the squirrel monkey found no relationship between sperm concentration or dibutyrl cAMP in culture medium and the level of triploidy or percent fertilization (Asakawa and Dukelow, 1982).

Human *in vitro* fertilization rates are quite high (70-90%) while the actual number of children delivered is low (10-25%). This increased embryonic and fetal death loss occurs after fertilization and might reflect the incidence of triploidy. Greater emphasis on research in this area is needed in order to reduce the frequency of triploidy.

MATERIALS AND METHODS

Animals

The animals used were adult squirrel monkeys (*Saimiri sciureus*) of Bolivian and Guyanese origin (South American Primates, Miami, Florida). They were housed indoors on a 12:12 hour light:dark cycle with room temperature maintained at 21 $\pm$ 3 ^{o}C. During the summer months (June to October), the animals were maintained in large colony cages outdoors (Jarosz and Dukelow, 1976). The animals were fed a commercial monkey chow (5047, Ralston-Purina Co., St. Louis, Missouri) supplemented with apple slices. Fresh water was available *ad libitum*.

Follicular Induction Regimens

Mature female squirrel monkeys received one of the following follicular induction regimens: standard FSH plus HCG, clomiphene citrate plus HCG, prostaglandin E1 with HCG, and prostaglandin E1 without HCG. The following protocol was used in the control FSH group. Female squirrel monkeys received a follicular induction regimen consisting of four daily i.m. injections of follicle stimulating hormone (1 mg, FSH-P^{R}, Burns-Biotec Laboratories Inc., Omaha, Nebraska) and a single i.m. injection of human chorionic gonadotropin (250 IU, HCG. A.P.L.^{R} Ayerst Laboratories, Inc. New York, New York) on the fourth day (Dukelow, 1970; 1979). Five daily FSH injections, rather than four, were given (Kuehl and Dukelow, 1975a) during the anovulatory months (July through September) followed by HCG.

Clomiphene citrate (Sigma Chemical Co., St. Louis, Missouri) was mixed with Gatorade^{R} (Stokely-Van Camp,Inc, Chicago,Illinois) and give orally to female squirrel monkeys at doses of either 2 mg, 4 mg or 6 mg per animal for four days followed by an intramuscular injection of HCG on the last day.

Prostaglandin E1 (2 mg, Sigma Chemical Co., St. Louis, Missouri) was tested by administration of FSH for four days with an injection of prostaglandin E1 on the afternoon of the fourth day given either with or without an injection of 250 IU of HCG.

Laparoscopic Recovery of Oocytes

The use of laparoscopy in reproductive studies has been extensively reviewed by Harrison and Wildt (1980). The laparoscopic technique for oocyte recovery in squirrel monkeys has been previously described (Dukelow et al., 1978; Dukelow and Ariga, 1976). The squirrel monkeys were anesthetized with sodium pentobarbital (27 mg/kg body

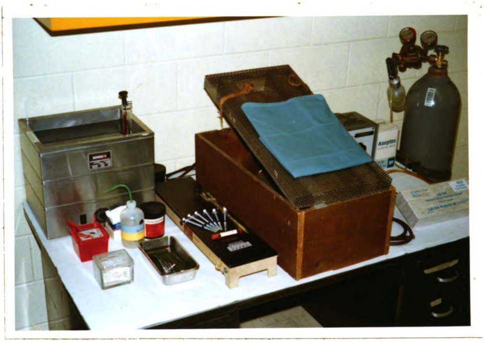

Figure 1. Laparoscopic equipment for oocyte retrieval

weight per adult female, i.m.) 15 to 16 hours after the HCG injection. A small midline incision was made with a scapel and the trocar-cannula inserted. The trocar was then removed and the laparoscope (4 mm diameter, Karl Storz Co., West Germany) inserted. To improve viewing, the abdominal cavity was insufflated with carbon dioxide passed through the cannula. A 25 gauge needle and 1 ml tuberculin syringe were used to move the fimbria aside exposing the ovaries. The ovarian follicles were counted according to size (large >3 mm; medium 3 mm to 1 mm; small <1 mm) and aspirated using a 25 gauge 5/8 inch needle. The oocytes were aspirated into 0.05 ml of TC-199 culture medium (with 25 mM Hepes Buffer, Earle's Salts and L-Glutamine, Gibco Laboratories, Ohio) supplemented with 20% heat inactivated GG-free bovine serum albumin (Gibco Laboratories), 1 mM pyruvate (Sigma Chemical Co., St. Louis, Missouri), 100 mg per ml Gentamicin sulfate (M.A. Bioproducts, Walkersville, Maryland) and 1 unit per ml heparin. The oocytes were placed into sterile 8-chamber tissue culture slides (Lab-Tek Products, Napierville, Illinois) and incubated at 37 ^{o}C in a moist atmosphere of 5% CO_2 in air. The cultures were observed every 24 hours with an inverted microscope. The numbers of oocytes collected and their stage of maturation was recorded for each monkey.

Semen Collection

The adult male squirrel monkey was restrained in a V-shaped table (Kuehl and Dukelow, 1974) and short pulses of current (120 pulses per second at 5 msec duration) were delivered to a probe placed inside the rectum. The voltage was gradually increased and decreased in a rhythmic pattern every 3 seconds. Sperm cells in the ejaculated coagulum plug were collected and held at 37 ^{o}C for 30 minutes to allow liquification. The sperm were evaluated for motility, percentage of progressive motility, percentage of immature sperm and sperm concentration. The sperm cells were then held at 37 ^{o}C for 5-10 minutes. The oocytes were inseminated with 0.05 ml of the sperm suspension at 20-21 hours after oocyte recovery (10^{5}-10^{6} sperm/ml). Oocytes mature at recovery were inseminated immediately.

Collection and Preparation of Homologous Serum

Blood samples were needed in order to compare the effect of squirrel monkey serum versus fetal calf serum in the culture medium on fertilization and growth of embryos *in vitro*. Samples were taken from the femoral vein of female squirrel monkeys and allowed to clot overnight. A 3 ml syringe fitted with a 25 gauge needle was used to draw 2-3 ml of blood. The clot was centrifuged for 30 minutes at 4 ^{o}C, the serum drawn off and stored at -20 ^{o}C in 3 ml vacutainers (Becton Dickinson, Rutherford, New Jersey). The

Figure 2. Equipment used in semen collection

serum was heat-inactivated for 30 minutes at 56 ^{o}C before addition to modified TC-199 culture medium.

Criteria of Maturation and Fertilization

At intervals of approximately 24 hours, the cultures were examined and the stage of development recorded. An oocyte was considered mature if the presence of one polar body was noted. The criteria for fertilization were:

1) two or more polar bodies observed in the perivitelline space
2) two or more polar bodies and two or more symmetrical blastomeres 24 hours after insemination
3) observation of the sperm midpiece or tail within the cytoplasm.

An oocyte that possessed one or more of the above characteristics was considered fertilized.

Polyspermy

An analysis of previous *in vitro* fertilization trials over the past 15 years was done. The incidence of triploidy versus sperm concentration was examined. Since a wide variety of experiments have been carried out, only those experiments which could be identified as controls were studied.

Culture Medium Analysis

Various properties of the culture medium were analyzed at 0, 24 and 48 hours after culture in the incubator. Starting osmolarity ranged from 280 to 300 mOsm and was determined using a freezing point osmometer (Osmette, Precision Systems, Inc., Natick, Massachusetts). Calcium concentration was analyzed using a Calcette ^{R} (Precision Systems, Natick, Massachusetts) at the various time intervals. This instrument measures total calcium concentration in aqueous solutions using a fluorometric titration technique. The pH of the medium was determined using a Model 501 digital ionalyzer (Orion Research Inc., Boston, Massachusetts) and a system 1304 pH/blood gas analyzer (Instrumentation Laboratory, Lexington, Massachusetts). The pCO_2, pO_2, bicarbonate content, total CO_2 content, standard bicarbonate and oxygen saturation at normal P50 were measured using the system 1304 pH/blood gas analyzer.

Statistical Analysis of Data

The Student-Newman-Keuls test and analysis of variance (ANOVA) were used to test the hypothesis that there was no difference between follicular development and induction regimens. A chi-square test was used to examine the hypothesis that fertilization rates were higher in the control group than the treatment groups. The relationship between *in vitro* maturation, fertilization and culture medium variables were evaluated using Pearson's linear correlation.

RESULTS

Comparision of Serum Source

Homologous serum (20%) pooled from several monkeys was substituted for fetal calf serum in TC-199 culture medium in order to evaluate its effect on *in vitro* fertilization and subsequent development. Oocytes were collected from hormonally-primed females and inseminated in culture medium containing either fetal calf serum or homologous serum as the protein source. There was no significant difference in fertilization rate ($p>0.05$) but the number of embryos cleaving to the two-cell stage was greater ($p<0.05$) when fetal calf serum was the protein source (Table 2). Table 3 shows the culture characteristics of fetal calf serum and homologous serum after analysis with the 1304 blood-gas analyzer. Bicarbonate concentration and standard bicarbonate content for homologous serum were twice that of fetal calf serum. The pH of fetal calf serum was neutral while homologous serum was slightly basic. The calcium concentration in fetal calf serum was double that of homologous serum but osmolarity, percent oxygen saturation, total carbon dioxide, and pO_2 were similar for the two serum types.

TABLE 2

Comparison of fertilization and early development of squirrel monkey oocytes in fetal calf serum or homologous serum in TC-199 culture medium

Factors	20% Fetal Calf Serum		20% Homologous Serum	
no. animals	19		22	
no. eggs collected	27		49	
no. atretic eggs	3	(11.1%)	6	(12.2%)
no. mature eggs at recovery	5	(21.0%)	10	(23.2%)
no. fertilized eggs	8		11 [a]	
no. fertilized/no. inseminated	8/24	(33.3%)	11/43	(25.6%)
no. two-cell embryos	4		2 [b]	

a $p>0.05$ not significant (Chi-square test)

b $p<0.05$ significant (Chi-square test)

TABLE 3

Characteristics of fetal calf serum and homologous serum after analysis with 1304 blood-gas analyzer

Characteristic	Fetal calf serum (mean±SD)	Squirrel monkey serum (mean±SD)
pH	7.00±0.04	7.67±0.02
PCO_2	39.9±4.31	16.1±3.56
PO_2	146±4.95	133±5.01
HCO_3^- (mmol/L)	10.1±0.07	18.8±0.09
TCO_2 (mmol/L)	11.3±0.21	19.3±0.30
SBC (mmol/L)	9.3±0.71	20.5±0.66
$\%SO_2c$ (mmol/L)	98.0±0.57	99.6±0.19
Osmolarity (mOsm)	309±1.41	304±0.04
Calcium (mEq/L)	6.98±0.23	4.30±0.28

TCO2: total carbon dioxide
SBC: standard bicarbonate content
%SO2c: percent oxygen saturation content

Follicular Induction

Three follicular growth regimens were examined and compared to the control FSH-HCG treatment. The results of follicular development and oocyte yield are shown in Table 4. Follicular growth of large and small follicles in monkeys receiving either 2 or 4 mg of clomiphene citrate was significantly lower ($p<0.05$) than the control group. Animals given 6 mg of clomiphene showed growth of follicles similar to those of the FSH-HCG group. There were significantly fewer large follicles in the prostaglandin E1 plus HCG group and smaller follicles in the prostaglandin E1 alone group ($p<0.05$) than in the controls. The lowest number of oocytes were obtained from animals given 2 or 4 mg clomiphene citrate and those administered prostaglandin E1 plus HCG. Significantly more atretic oocytes were recovered from the two low doses of clomiphene ($p<0.01$) and the high dose of clomiphene citrate ($p<0.05$) when compared to the controls. Recovery of mature oocytes at laparoscopy was greater in animals administered prostaglandin E1 and HCG, but fewer mature ova were collected from monkeys given 2 or 6 mg of clomiphene citrate ($p<0.05$). A mature oocyte collected from a monkey in the 4 mg clomiphene group 16 hours after HCG is shown in Figure 4. The largest number of oocytes maturing *in vitro* were from monkeys given 6 mg of clomiphene citrate ($p<0.05$).

Figure 3. Mature squirrel monkey oocyte recovered 16 hours after administration of hCG

TABLE 4

Follicular development of squirrel monkeys receiving induction regimens

Trial:	2mg CC HCG	4mg CC HCG	6mgCC HCG	PGE1 HCG	PGE1 alone	FSH-HCG (control)
no. of Monkeys	20	20	22	20	20	23
Mean no. of Follicles						
Large	0.7a	0.8a	1.0	0.7a	1.4	1.1
Medium	2.0	2.7	3.6	2.8	3.6	3.6
Small	6.0a	5.4a	8.9	6.7	5.1a	8.3
no. of oocytes	8	20	44	24	40	43
no. Atretic oocytes	4[c]	10[c]	13[b]	4	7	3
(%)	(50)	(50)	(29)	(16)	(17)	(7)
no. Mature at recovery	0[a]	6	5[a]	16	18	15
(%)	(0)	(60)	(16)	(80)	(54)	(38)
no. Mature (37 h. post HCG)	1	2	7[a]	1	1	1
(%)	(25)	(50)	(27)	(25)	(7)	(7)
no. oocytes per monkey	0.4	1.0	2.0	1.2	2.0	1.8

[a] significantly different from control ($p<0.05$) (Students t-test)

[b] significantly different from control ($p<0.05$) (Chi-square)

[c] significantly different from control ($p<0.01$) (Chi-square)

In Vitro Fertilization

Oocytes obtained from all of the follicular induction regimens were able to be fertilized (Table 5) and there was no difference between the treatment groups and control for fertilization rate. No embryos cleaved beyond the two-cell stage in the 2 or 4 mg clomiphene citrate groups while development proceeded to the 4 to 8 cell stage in the remaining groups. A two-cell embryo from the control group found 30 hours after insemination is shown in Figure 4.

Culture Medium Analysis

Culture medium characteristics over time for the follicular induction regimens are shown in Tables 6 through 11. The pCO_2 and pO_2 changed the most throughout all experiments while pH, bicarbonate concentration, TCO_2, standard bicarbonate content, percent oxygen saturation and osmolarity remained stable over the 48 hour culture period. There was a strong inverse relationship between *in vitro* maturation rate and low calcium concentration ($r^2=0.98$) while a direct relationship was found for fertilization rate and calcium content ($r^2=0.90$). No correlation between maturation or fertilization rates and the remaining culture characteristics was found.

TABLE 5

Fertilizability of oocytes recovered from squirrel monkeys receiving follicular induction regimens

Trial:	2mg CC HCG	4mg CC HCG	6mgCC HCG	PGE1 HCG	PGE1 alone	FSH-HCG (control)
no. of Monkeys	20	20	22	20	20	23
no. of oocytes	8	20	44	24	40	43
no. Fertilized	1	6	14	11	14	14
(%)	(100)	(75)	(85)	(65)	(74)	(87)
no. Developed Beyond Two-Cell Stage	0	0	3	1	1	2
(%)	(0)	(0)	(21)	(9)	(7)	(14)
no. viable oocytes per monkey	0.2	0.5	1.4	1.0	1.7	1.7

[a] $p<0.05$ significant from control (Chi-square)

[b] $p<0.01$ significant from control (Chi-square)

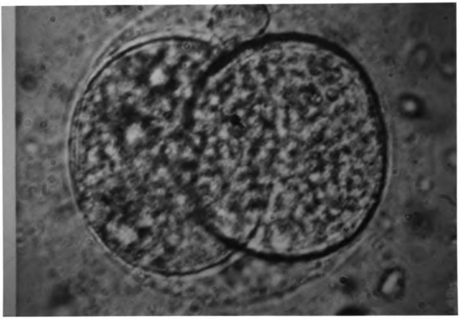

Figure 4. Two-cell squirrel monkey embryo 30 hours after insemination

TABLE 6

Culture medium characteristics over time for embryos recovered following follicular induction with 1 mg FSH-HCG

Characteristic	Time 0 pre-culture (mean+SD)	24 hours (mean+SD)	48 hours (mean+SD)
pH	7.38±0.07	7.39±0.01	7.53±0.01
pCO_2	34.7±0.07	33.3±0.49	37.0±1.27
pO_2	165±0.00	118±0.71	115±1.41
HCO_3^{2-} (mmol/L)	20.8±0.07	20.5±0.07	20.8±0.21
TCO_2 (mmol/L)	21.8±0.00	21.5±0.07	21.9±0.14
SBC (mmol/L)	22.6±0.00	22.6±0.14	22.1±0.14
$\%SO_2c$ (mmol/L)	99.5±0.00	98.6±0.00	98.3±0.14
Osmolarity (mOsm)	315±0.71	302±0.00	304±0.71
Calcium (mEq/L)	3.83±0.57	4.01±0.06	4.08±0.04

TCO2: total carbon dioxide
SBC: standard bicarbonate content
%SO2c: percent oxygen saturation content

TABLE 7

Culture medium characteristics over time for embryos recovered following follicular induction with 2 mg clomiphene citrate-HCG

Characteristic	Time 0 pre-culture (mean±SD)	24 hours (mean±SD)	48 hours (mean±SD)
pH	7.43±0.05	7.61±0.03	7.46±0.1
pCO_2	34.7±1.30	20.4±2.30	30.7±7.1
pO_2	189±1.60	145±6.90	151±5.6
HCO_3^- (mmol/L)	23.2±1.80	20.4±1.00	21.8±0.3
TCO_2 (mmol/L)	24.3±1.70	21.0±1.00	22.7±0.2
SBC (mmol/L)	24.9±1.90	26.4±0.02	24.7±2.1
$\%SO_2c$ (mmol/L)	99.7±0.06	99.6±0.01	99.5±0.2
Osmolarity (mOsm)	294±0.70	311±25.0	306±7.2
Calcium (mEq/L)	-	-	-

TCO2: total carbon dioxide
SBC: standard bicarbonate content
%SO2c: percent oxygen saturation content

TABLE 8

Culture medium characteristics over time for embryos recovered following follicular induction with 4 mg clomiphene citrate-HCG

Characteristic	Time 0 pre-culture (mean±SD)	24 hours (mean±SD)	48 hours (mean±SD)
pH	7.48±0.02	7.75±0.03	7.72±0.05
pCO_2	22.15±5.00	11.75±0.90	12.28±1.02
pO_2	153.3±11.8	127.8±8.90	129.5±10.1
HCO_3^- (mmol/L)	16.6±3.00	16.2±2.70	16.45±2.95
TCO_2 (mmol/L)	17.3±3.20	16.5±2.70	16.83±2.98
SBC (mmol/L)	21.8±1.60	26.4±2.20	25.85±2.73
$\%SO_2c$ (mmol/L)	99.6±0.06	99.7±0.06	99.5±0.06
Osmolarity (mOsm)	295±8.70	302±0.70	303±2.98
Calcium (mEq/L)	3.3±0.50	3.2±0.70	3.2±0.00

TCO2: total carbon dioxide
SBC: standard bicarbonate content
%SO2c: percent oxygen saturation content

TABLE 9

Culture medium characteristics over time for embryos recovered following follicular induction with 6 mg clomiphene citrate-HCG

Characteristics	Time 0 pre-culture (mean$\pm$SD)	24 hours (mean$\pm$SD)	48 hours (mean$\pm$SD)
pH	7.45$\pm$0.00	7.50$\pm$0.07	7.52$\pm$0.04
pCO_2	32.7$\pm$1.30	29.1$\pm$6.90	27.6$\pm$4.40
pO_2	188$\pm$2.20	146$\pm$3.00	147$\pm$7.60
HCO_3^- (mmol/L)	23.3$\pm$0.70	22.8$\pm$2.00	22.5$\pm$1.80
TCO_2 (mmol/L)	23.9$\pm$0.22	23.7$\pm$2.20	23.4$\pm$2.00
SBC (mmol/L)	25.6$\pm$1.00	26.1$\pm$0.40	26.2$\pm$0.50
$\%SO_2c$ (mmol/L)	99.7$\pm$0.00	99.6$\pm$0.20	99.5$\pm$0.20
Osmolarity (mOsm)	282$\pm$1.51	294$\pm$8.00	294$\pm$9.33
Calcium (mEq/L)	-	-	-

TCO2: total carbon dioxide content
SBC: standard bicarbonate content
%SO2c: percent oxygen saturation content

TABLE 10

Culture medium characteristics over time for embryos recovered following follicular induction with 2 mg prostaglandin E1 without HCG

Characteristics	Time 0 pre-culture (mean$\pm$SD)	24 hours (mean$\pm$SD)	48 hours (mean$\pm$SD)
pH	7.43$\pm$0.03	7.48$\pm$0.06	7.57$\pm$0.05
pCO_2	29.1$\pm$0.90	25.0$\pm$5.62	16.6$\pm$6.91
pO_2	171$\pm$17.0	146$\pm$2.93	165$\pm$8.22
HCO_3^{2-} (mmol/L)	19.5$\pm$0.61	18.7$\pm$1.60	14.8$\pm$4.50
TCO_2 (mmol/L)	20.4$\pm$0.53	19.5$\pm$1.71	15.3$\pm$4.73
SBC (mmol/L)	22.7$\pm$0.82	23.2$\pm$0.20	22.3$\pm$1.80
$\%SO_2c$ (mmol/L)	99.6$\pm$0.15	99.5$\pm$0.15	99.7$\pm$0.13
Osmolarity (mOsm)	285$\pm$3.20	280$\pm$9.30	294$\pm$4.64
Calcium (mEq/L)	3.87$\pm$0.11	3.90$\pm$0.04	3.94$\pm$0.07

TCO2: total carbon dioxide
SBC: standard bicarbonate content
%SO2c: percent oxygen saturation content

TABLE 11

Culture medium characteristics over time for embryos recovered following follicular induction with 2 mg prostaglandin with HCG

Characteristic	Time O pre-culture (mean±SD)	24 hours (mean±SD)	48 hours (mean±SD)
pH	7.46±0.03	7.38±0.06	7.34±0.05
pCO_2	25.6±4.61	31.6±1.80	35.1±1.11
pO_2	159±8.10	133.3±13.7	132.5±14.5
HCO_3^- (mmol/L)	18.27±2.13	18.95±1.85	19.45±2.02
TCO_2 (mmol/L)	17.8±4.32	19.9±1.83	20.5±2.00
SBC (mmol/L)	22.5±0.81	21.5±2.21	21.2±2.14
$\%SO_2c$ (mmol/L)	99.5±0.12	99.0±0.19	98.9±9.83
Osmolarity (mOsm)	292±6.90	296±7.93	301±9.81
Calcium (mEq/L)	3.87±0.11	3.90±0.04	3.94±0.07

TCO2: total carbon dioxide
SBC: standard bicarbonate content
%SO2c: percent oxygen saturation content

Polyspermy

A retrospective study of 28 previous control *in vitro* fertilization experiments found the incidence of polyspermy to be 11% (Table 12). The culture medium used in all the experiments was TC-199 supplemented with 20% fetal calf serum and pH ranged from 7.3 to 7.8. A total of 107 oocytes were fertilized (60%) and 12 ova were found to be polyspermic. Polyspermy occurred over a range of sperm concentrations (1.4×10^4 / ml to 4.7×10^6 /ml) and there was no correlation found between the incidence of polyspermy and sperm concentration.

TABLE 12

Incidence of polyspermy in the squirrel monkey *in vitro* fertilization system

no. of experiments	28
no. of oocytes	486
no. of mature oocytes (16 hours post HCG)	179 (37%)
no. of fertilized oocytes	107 (60%)
no. of polyspermic oocytes	12 (11%)
average no. sperm/ml used for insemination	5.1×10^5
average no. sperm/ml of non-polyspermic oocytes	3.4×10^5
average no. sperm/ml of polyspermic oocytes	8.8×10^5

DISCUSSION

These studies indicate that the squirrel monkey is a viable model for examining various aspects of *in vitro* fertilization, such as follicular induction regimens, culture conditions and polyspermy. Induction of follicular growth has been accomplished in the squirrel monkey with pregnant mares serum (Bennett, 1967; Gould et al., 1973), porcine follicle-stimulating hormone (Dukelow, 1970) and human urinary follicle-stimulating hormone (Yano and Gould, 1985). Clomiphene citrate has been successfully used to induce follicular growth in the baboon (Irsigler et al., 1983) and humans (Holman and Hammond, 1988).

This study examined three doses of clomiphene citrate for inducing follicular growth and oocyte yield in the squirrel monkey. Oocyte production with 6 mg (8 mg/kg bwt) of clomiphene citrate for four days showed similar effectiveness as the standard FSH-HCG regimen. Irsigler et al. (1984) used a dose of 5 mg/kg for follicle induction in baboons and reported collecting an average of five oocytes per animal. Of those ova, 20% were atretic, 51% were mature and the remaining immature. In chimpanzees, one oocyte was aspirated per monkey after administration of 1.4 mg/kg clomiphene citrate for five days (Gould, 1983). The present study examined doses of 3, 6 and 8 mg/kg body weight and 0.4, 1 and 2 oocytes per animal were collected, respectively. Gould (1983) reported growth of one large follicle in 58% of the animals and development of one or more follicles in 42% of the cases. This is similar in the squirrel monkey with an average of one follicle growing per animal but there was development of medium and small size follicles in almost all of the animals.

There were significantly more atretic oocytes collected from animals given clomiphene citrate. Although the exact cause is not known, Littman and Hodgen (1985) speculated that elevated luteinizing hormone/HCG levels may increase androgen production by the theca cells, thereby promoting folliclular atresia. It is unclear whether clomiphene citrate acts on the hypothalamus, pituitary or on the ovary to cause this early rise in luteinizing hormone which has been observed in cynomolgus monkeys.

There were significantly fewer follicles (large and small) with the two lowest doses of clomiphene citrate. Marrs et al. (1984) reported that clomiphene alone may not provide enough gonadotropin support for multiple follicle development. For this reason, Balmaceda et al. (1984) chose to use human menopausal gonadotropin over clomiphene since HMG yielded a larger number of mature oocytes at aspiration.

There was little difference in follicle growth and oocyte production in the prostaglandin E1 treatments groups. In the control group, 38% of the ova were mature at collection which is similar to previous reports for this regimen (Kuehl and Dukelow, 1975). However, when prostaglandin E1 and HCG were administered together, significantly more mature oocytes were aspirated at laparoscopy. This study reports 80% of the oocytes collected after prostaglandin-HCG treatment were mature (Table 4). A previous study (Batta and Brackett, 1978) found 75% of the oocytes retrieved were mature, which is comparable to results reported here. The authors ascribed this effect to the action of prostaglandin E1 and HCG for providing the primary stimulus for the resumption of meiosis resulting in oocyte maturation. This synchronization was observed with the squirrel monkeys when both PGE 1 and HCG were administered. While prostaglandin E1 alone increased maturation, it was not significanly different from the control.

Oocytes collected from all of the follicular induction regimens were successfully fertilized. There was no significant difference in fertilization rate between the treatment and control groups. A fertilization rate of 87% was achieved in the control FSH-HCG group which is comparable to results described earlier for the squirrel monkey (Chan et al., 1982). Fertilization rates of oocytes aspirated after administration of clomiphene citrate were slightly higher than in previous work (Gould, 1983; Irsigler et al., 1984). In the baboon, low fertilization was attributed to difficulty in semen collection and processing as well as culture contamination.

Polyspermy is known to be a more common occurrence *in vitro* than *in vivo*. A retrospective study was done to examine the incidence of polyspermy and sperm concentration in our *in vitro* fertilization system. High sperm concentrations are used in human *in vitro* fertilization systems (Lopata et al., 1980; Trounson et al., 1981) and it was thought that the large concentration of sperm might account for this observation. Wolf et al. (1984) described increased polyspermic fertilization (5.5%) at higher sperm concentrations (10^5 sperm/ml) as compared to no polyspermic embryos when 10^4 sperm/ml were used to inseminate human ova. No relationship between polyspermy and sperm concentration (within the range of 1.3×10^5 to 5×10^5) in humans was reported by Diamond et al. (1985a,b). The present study found no correlation between sperm concentration and polyspermy since ova became polyspermic with concentrations ranging from 1.4×10^4 to 4.7×10^6 sperm/ml. The incidence of polyspermy in the control experiments examined was 11% which is comparable to a previous report (Asakawa and Dukelow, 1982). Irsigler reported polyspermy in only 2 out of 90 baboon oocytes fertilized *in vitro*.

This work examined culture medium characteristics over a period of 48 hours and any relationship to *in vitro* maturation and fertilization. Tables 6 through 11 document culture medium values for the previous follicular induction trials. The basic medium used in all of the experiments was modified TC-199 (with Earles salts) supplemented with 20% fetal calf serum, sodium pyruvate, gentamicin and heparin. Initial pH and osmolarity were adjusted to 7.4 to 7.5 and 280-300 mOsm, respectively. The pH remained fairly stable when incubated in a 5 % CO_2-moist air atmosphere. This is in agreement with Bavister (1981) who stated that bicarbonate-CO_2 buffer system yielded the best results for fertilization in hamsters. The pH in those experiments decreased from 7.6 to 7.5 with TALP medium while in the current squirrel monkey system, pH generally increased from 7.4 to 7.5 in the TC-199. The optimal pH for *in vitro* fertilization in hamsters is 7.4 (Bavister, 1981) with higher pH levels leading to increased incidence of polyspermy (Bavister, 1969). However, the pH of squirrel monkey serum (Table 3) was 7.67 so a slight increase in pH from 7.5 to 7.6 would probably not be detrimental. The fact that the pH of the TC-199 cultured in a bicarbonate-CO_2

system remains stable over a period of at least 48 hours is encouraging.

The osmolarity of the medium also did not change significantly over time. A wide range of osmotic pressures have been reported to support *in vitro* fertilization (Miyamoto and Chang, 1973; Bae and Foote, 1983). The latter group of researchers described the highest *in vitro* maturation of rabbit oocytes at 270 mOsm but this was not significantly different for osmotic pressures ranging from 250-310 mOsm. No relationship between osmolarity and *in vitro* maturation rate was observed in the squirrel monkey study.

Oxygen saturation (%SO_2c) and total carbon dioxide content (TCO_2) remained quite constant throughout time in these experiments. The normal human values are 96-97% and 23-27 mmol/L (Altman, 1961), respectively while values for squirrel monkey serum were comparable at 19.3 ± 0.3 mmol/L and 99.6 ± 0.2% (Table 3). Actual bicarbonate content and standard bicarbonate content (SBC) found for squirrel monkey serum were 18.8 ± 0.1 mmol/L and 20.5 ± 0.7 mmol/L which is slightly lower than reported in humans (22-26 mmol/L and 22-26 mmol/L), respectively (Altman, 1961). These values remained stable over time in the follicular induction experiments which aided in stablilizing the pH of the medium.

The pCO_2 and pO_2 did vary somewhat throughout the 48 hour period. In all of the experiments except the 4 mg of clomiphene and prostaglandin E1 alone, the percent of carbon dioxide usually decreased from 5 % to 4 %. The starting pCO_2 in the two aforementioned experiments was low (3-4% and dropped down to 1.7 and 2.2 %, respectively). However, it did not seem to have an adverse effect on *in vitro* maturation or fertilization. The average starting pO_2 for all six experiments was 168 mmol/L (23% oxygen atmosphere) and decreased to 135 mmol/L (19% oxygen) after 48 hours. The oxygen atmosphere in this study is within the acceptable range reported in other experiments (Wright et al., 1976; Peters et al., 1977). The embryos utilize oxygen in the oxidation of pyruvate so oxygen tension would be expected to decrease (Betterbed and Wright, 1984).

Calcium is known to play an important role in reproduction (acrosome reaction, sperm binding, oocyte maturation and cell division). Calcium concentrations ranged from 3.2 to 4.0 mEq/L in the six follicular induction experiments. An inverse relationship was found between *in vitro* maturation and calcium levels (r^2=0.98) suggesting that high calcium concentrations in culture may have an adverse effect on maturation. It is also possible that magnesium levels were too low since a certain ratio of calcium to magnesium may be required

(Bavister, 1981) for maturation and fertilization, however, magnesium was not measured in this experiment. There was also a correlation found between fertilization rate and calcium concentration (r^2=0.90). This could explain the observation that supplementing TC-199 medium with fetal calf serum rather than squirrel monkey serum resulted in a higher fertilization rate. However, there might be factors present in squirrel monkey serum that were not examined in this study that could contribute to the high correlation that was observed. There might be certain factors present in the serum that are embryotoxic or the concentration of serum used may have been too high. Another possible explanation for the high degree of correlation observed could be due to the low number of observations. More work in this area is needed to determine an optimal concentration of serum or even if blood serum is necessary. Some other *in vitro* fertilization systems use bovine serum albumin (Bavister et al., 1983; Balmaceda et al., 1984) instead of blood serum. Yanagimachi (1972) reported that serum albumin was not necessary but beneficial for fertilization of guinea pig oocytes *in vitro*.

A better understanding of optimal *in vivo* conditions and more precise culture techniques should aid in increasing mammalian *in vitro* fertilization rates in species where *in vitro* fertilization has been successful and perhaps in species where fertilization has been sporadic or not reported at all.

SUMMARY AND CONCLUSIONS

The squirrel monkey *in vitro* fertilization system is useful in studying early embryo development, culture conditions and physiological phenomenon such as polyspermy. The following conclusions resulted from the data obtained:

1) Homologous serum is an adequate protein source in TC-199 fertilization medium for squirrel monkey oocytes but more embryos cleave to the two-cell stage when fetal calf serum is used as the protein source. The calcium concentration in the serum could account for the difference.
2) Induction of follicular growth with 2 and 4 mg of clomiphene citrate was poor. Administration of 6 mg of clomiphene citrate for four days provided similar follicular growth as FSH-HCG.
3) Injection of animals with prostaglandin E1 or prostaglandin E1 plus HCG resulted in more mature oocytes after laparoscopy. The prostaglandin E1 did not enhance follicular growth but did synchronize oocyte maturation.
4) Oocytes resulting from the follicular induction regimens were able to be fertilized and develop beyond the two-cell stage in most cases.

5) There was a strong inverse relationship between *in vitro* maturation and low calcium concentration of the medium while fertilization was higher when calcium concentration was greater.

6) The incidence of polyspermy found in the experiments examined in this study was 11%. Oocytes became polyspermic in sperm concentrations ranging from 1.4×10^4 sperm/ml to 4.7×10^6 sperm/ml. There was no relationship between sperm concentration and the incidence of polyspermy.

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APPENDICIES

APPENDIX

PUBLICATIONS BY THE AUTHOR

Full Papers

1) Antigenic profile and impact of immunization with zona pellucida antigens in primates. A.G. Sacco, M.G. Subramanian, E.C. Yurewicz, D.L. Pierce and W.R. Dukelow. In: Immunological Approaches to Contraception and Promotion of Fertility. (G.P. Talwar, ed.) Plenum Press. New York. pp. 277-290 1986.

2) Ovaries remain functional in squirrel monkeys (*Saimiri sciureus*) immunized with porcine zona pellucida 55,000 macromolecule. Anthony G. Sacco, Donna L. Pierce, Marappa G. Subramanian, Edward C. Yurewicz and W. Richard Dukelow. Biol Reprod. 36, 481-490. 1987.

3) *In vitro* fertilization in nonhuman primates. W. Richard Dukelow, D.L. Pierce, W.E. Roudebush, S.J. Jarosz and K. Sengoku. J. Med. Primat. (in press).

Abstracts

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3) Laparoscopic observation of the mouse uterus. W.R. Dukelow, D.L. Pierce, J.A. Kontio and L. Martin. Proc. Michigan Academy of Science.1985.

4) The effect of a ZP-3 antigen vaccine on the fertility of squirrel monkeys (*Saimiri sciureus*). D.L. Pierce, A.G Sacco, M.G. Subramanian and W.R. Dukelow. American J. Primatol. 8, 357. 1985.

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7) *In vitro* fertilization in nonhuman primates. W. R. Dukelow, W.E. Roudebush, D.L. Pierce, J.K. Graham and K. Sengoku. Internat. J. Primatol. 1986.

8) Immunization of squirrel monkeys with porcine zona pellucida (ZP); effect on ovarian function. A.G. Sacco. M.G. Subramanian, E.C.Yurewicz, D.L. Pierce and W.R. Dukelow. Proc. 3rd International Congress of Reproductive Immunology. 1986.

9) Ovarian function in the squirrel monkey following immunization with porcine zona pellucida (ZP). A.G. Sacco, M.G. Subramanian, E.C. Yurewicz, D.L. Pierce and W.R. Dukelow. Amer. J. Primatol. 10, 1986.

10) Blastomere isolation and culture of preimplantation embryos. W.E. Roudebush, D.L. Pierce, K. Sengoku, W.R. Dukelow. Fertil. Steril. 48, 24. 1987.

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12) Induced cell loss: effects on the potential for embryos to develop in vitro. W.E. Roudebush, S.D. Kholkute, D.L. Pierce and W.R. Dukelow. Proc. Society for the Study of Reproduction. 1988.

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