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In vitro fertilization and preimplantation development in the primate

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Abstract. Nonhuman primates represent a strong model for examining the chromosomal, biochemical, and temporal normality of embryos produced by *in vitro* fertilization. More *in vitro* fertilized embryos from the squirrel monkey (*Saimiri sciureus*) have been produced and examined than with all other primate species combined. In studies over a 13 year period a fertilization rate approximating 60 % has been developed in this species with 30% of these embryos proceeding to the two cell stage and 50% of these to the three-four cell stage. Chromosomal abnormalities (primarily missing or extra chromosomes) at a level of nine to 16% have been found, a value corresponding to that found in *in vivo* mating and *in vitro* fertilization in other species. An incidence of triploidy of 16.7% was observed. RNA and protein synthetic rates appear comparable with those of laboratory species subjected to *in vitro* fertilization and indicate the initial stages of metabolic activity of the newly formed embryo. Similarly, increases in estrogen incorporation appear after fertilization but no effect is observed in progesterone incorporation. Utilizing 2-deoxy-glucose and insulin, it was determined that the glucose requirement as an energy source for early preimplantation *in vitro* fertilized primate embryos is very low.

Of very great importance is the temporal relationship of the development of *in vitro* fertilized squirrel monkey embryos compared with similar development in other primates (including humans) after *in vivo* and *in vitro* fertilization. An analysis of over a decade of work with the squirrel monkey embryos demonstrates a pattern of temporal development that is comparable with all other primate species that have been examined (including the human) and comparable with development after *in vivo* fertilization.

Keywords. Fertilization; implantation; primate; embryos.

Introduction

In vitro fertilization in laboratory animals was first achieved in the 1950's, but it required over 20 years before this technique was successful with humans. Since the first human birth in 1978, a large number of in *vitro* fertilization clinics have developed around the world and several hundred pregnancies have been established. Because of ethical and moral constraints it is impossible to subject *in vitro* fertilized human embryos to many of the common cytogenetic and biochemical assays to determine normality. While none of the children born of this procedure have yet shown abnormal types of development, it is desirable that scientific studies be undertaken, using nonhuman primates as a model, on the development of embryos produced by *in vitro* fertilization.

Several research groups have reported successful *in vitro* fertilization in the nonhuman primate. The efforts of our laboratory were first fully reported in 1975 (Dukelow and Kuehl, 1975; Kuehl and Dukelow, 1975a). Since that time a number of

Abbreviations used: FSH, Follicle stimulating hormone; hCG, human chorionic gonadotropin; 2-DG, 2-deoxy-D-glucose.

publications have appeared from our laboratory relating to *in vitro* fertilized primate oocytes and were recently summarized (Dukelow *et al.,* 1983).

To successfully achieve a pregnancy it is necessary to not only *in vitro* fertilized the oocyte but also to effectively transfer the embryo to a recipient female. The first surgical transfer of an *in vivo* fertilized embryo was accomplished in the baboon by Kraemer *et al.* (1976). The first nonsurgical transfer was carried out by Pope *et al.* (1983) in the baboon. This involved an in *vivo* fertilized embryo. Finally, on July 25,1983, exactly five years after the birth of the first human in *vitro* fertilized embryo transfer baby, a baboon was born by this procedure (Kuehl *et al*., 1983). At the time of the writing of this manuscript, another baboon is pregnant as a result of transfer of an *in vivo* fertilized embryo that had been frozen, stored, and thawed (Dr. C. E. Pope, personal communication).

Thus it would appear that all of the procedures necessary for examining the normality of nonhuman primate offspring resulting from *in vitro* fertilization are available and tested. In addition to the research groups already cited, others have reported preliminary studies indicating successful *in vitro* fertilization in the rhesus monkey (Bavister *et al*., 1983), the cynomolgus macaque (Kreitmann *et al*., 1982) and the chimpanzee (Gould, 1983).

Materials and methods

All of the present studies were carried out with adult squirrel monkeys (*Saimiri sciureus*) of Brazilian or Guyanan origin. During the winter months the animals were housed in stainless steel, flush type cages which conformed to state and federal regulations. During the summer months the animals were housed out-of-doors in a cage system previously described by Jarosz and Dukelow (1976). The colony was composed of 140 females and 9 males. They received a commercial monkey diet with supplemental apples. Fresh water was supplied *ad libitum.* The animal room had fluorescent lighting on a 12L: 12D cycle and was temperature controlled at 21 ±2°C.

Because the estrous cycle of a squirrel monkey is not evident (there is no menstruation and vaginal cytology is not precise enough to predict ovulation), follicular development was induced in the animals by a regimen of four days of follicle stimulating hormone (FSH) (1 mg per day) followed by a single injection of 250 IU of human chorionic gonadotropin (hCG, APL,® Ayerst Company, Montreal, Canada). This regimen was previously published (Dukelow, 1970) and has been used in our laboratory for 15 years. During the summer months, because of the seasonal responsiveness of the squirrel monkey (Harrison and Dukelow, 1973) the period of FSH injection was extended to five days (Kuehl and Dukelow, 1975b). The minimum effective dose of hCG to induce ovulation has previously been determined to lie between 100 and 250 IU hCG (Dukelow, 1979). Semen was collected by the restraint and ejaculation procedure previously described (Kuehl and Dukelow, 1974). A coagulated ejaculate was incubated 30 min at 37°C and the resulting sperm suspension used to inseminate oocyte cultures.

Oocytes were recovered 16h after the hCG injection. This was accomplished laparoscopically (Dukelow and Ariga, 1976). The oocytes were aspirated into 0·1 ml of culture media and allowed to mature for 21 h prior to the addition of sperm. The

culture media used was TC 199 supplemented with 1 mmol pyruvate, 100 mg/ml of gentamicin and 1 IU/ml heparin. Twenty per cent fetal calf serum that had been heat-inactivated for 30 min at 56°C was added.

Oocyte maturation *in vitro* follows a linear pattern for about 40h after recovery. By 21h of culture about 50% of the oocytes are matured (Chan *et al.*, 1982).

The recovered oocytes with associated cumulus cells, follicular fluid and media were placed in tissue culture chamber slides. These were incubated at 37°C in a moist atmosphere of 5% CO_2 in air and checked at intervals for evidence of fertilization.

To assess the chromosomal normality of oocytes and embryos produced by *in vitro* fertilization, a final concentration of 0·04 gm/ml of colchicine was dissolved in some culture chambers 12 h after insemination. Oocytes were examined for the presence of polar bodies after removal of the cumulus cells. The oocytes or embryos were then prepared for chromosome analysis by a gradual fixation method with moist air (Mizoguchi and Dukelow, 1981). The oocytes were incubated in 0.1% trypsin in TC199 medium for 5 min to soften the zonae pellucidae. For hypotonic treatment the oocytes were incubated in 35% newborn calf serum for 30 min at 37°C. All preparations of oocytes and embryos were stained with 2% giemsa.

To assess the RNA synthetic rate, oocytes or embryos were incubated for three hours in modified TC199 medium with 2 0 % fetal calf serum supplemented with 5-[^{3}H]-uridine (final concentration 2·8 μM: specific activity, 18 Ci/mmol). This concentration allowed maximum incorporation to take place. The embryos were washed 10 times in nonradioactive medium, solubilized, and trichloroacetic acid-precipitable and non-precipitable material counted. Additionally, some embryos were fixed in Bouin's fluid after incubation with [^{3}H]-uridine and processed for autoradiography. The relative amount of uridine incorporation into RNA was determined by counting the reduced silver grains over a 300 μM^2 area of nucleoplasm or cytoplasm. These counts were converted to 1000 μM^2 for ease in calculation and graphic representation.

To assess the protein synthesis, autoradiographic procedures were used with the culture media supplemented with 0·4 μM L-4,5-[^{3}H]-leucine (specifc activity, 50·4 Ci/mmol). Incubation took place for 3h. The oocytes or embryos were then washed ten times with a medium containing unlabelled leucine, fixed 24 h in Bouin's fluid and processed for autoradiography. In addition, the ova were incubated for 2 h in 0·2 ml of culture medium with 0·06 μM of either labelled estradiol or progesterone. Ova were washed ten times in phosphate buffers, dissolved in 0·1 ml of tissue solubilizer, and the solutions assayed for radioactivity.

Additionally studies with 2-deoxy-D-glucose (2-DG) were carried out to assess the effects of insulin addition to the media and assess the importance of glucose as an energy source to early preimplantation primate embryos. Oocytes or *in vitro* fertilized embryos were preincubated for 1 h in 0·2 ml of media containing 5·56 mM D-glucose and washed five times. They then were incubated in media containing 2-deoxy-D-l-[^{3}H]-glucose (specific activity, 25 Ci/mmol). Accumulation over a 3h culture period was measured with and without the addition of 10 nM or 1 μM insulin from bovine pancreas. Following incubation the embryos or oocytes were washed ten times, solubilized, and radioactivity measured.

Finally, the rate of development of embryos in *in vitro* culture was determined (from all collected data over a ten year period) to provide a temporal scale of development.

This was compared with known values for both *in vitro* and *in vivo* fertilized embryos in the rhesus monkey, cynomolgus monkey, baboon and human.

Results

In the most recent summary, 995 oocytes have been recovered laparoscopically from the squirrel monkey. Of these 628 (31·5%) matured and 339 (54·0%) fertilized *in vitro.* In studies completed in 1981 and 1982 the *in vitro* fertilization rate approximated 60% and 30 % of these developed *in vitro* to the two cell stage. Of these 52% advanced to the three-four cell stage and embryos have been developed to the 16 cell stage. The latter have been subjected to electron microscopic evaluation (Yorozu *et al*., 1984). Electron-microscopic analysis indicates normality of the embryos.

Examining nonfertilized oocytes for chromosomal normality 666 oocytes were recovered from 2150 follicles. Of those oocytes assessed at the metaphase II stage from 7·4 to 14·0% exhibited abnormalities, normally missing or extra chromosomes. This value is comparable to that found in other laboratory species. Similar studies were carried out with oocytes exposed to *in vitro* fertilization conditions. Results are shown in table 1. Again, the incidence of abnormalities ranged from nine to 16% with the common abnormalities being missing or extra chromosomes. These values are also comparable to that found with natural fertilization and *in vitro* fertilization in a variety of laboratory species. Of interest was a relatively high incidence of triploidy of 16·7%. Triploidy has been reported to be a common problem with *in vitro* fertilization systems in other species including the human.

Table 1. Chromosomal abnormality incidence in *in vitro* fertilized squirrel monkey oocytes.

Developmental stage	Incidence of abnormality
Metaphase I	3/18 (16·7%)
Anaphase I	0/5
Metaphase II	
Haploidy	5/72 (8·9%)
Polarbody	4/29 (13·8%)
First cleavage metaphase	
Aneuploidy	3/25 (12·0%)
Triploidy	5/30 (16·7%)

RNA synthesis rate as measured by [^{3}H]-uridine incorporation is shown in table 2. Autoradiographic analysis demonstrated a significant increase in RNA synthetic capacity, after *in vitro* fertilization and again after the second cleavage division had begun. [^{3}H]-Leucine incorporation by a squirrel monkey oocyte decreased after a 21 h maturation period. The grain counts remained the same after in *vitro* fertilization with a nonsignificant elevation at the first cleavage (table 3).

Steroid uptake was analyzed for embryos fertilized *in vitro* and there was a trend for increased estradiol uptake with *in vitro* fertilization and first cleavage (table 4) although

Table 2. [^{3}H]-Uridine incorporation by *in vitro* fertilized squirrel monkey ova.

Cell stage	*n*	No. of grains/1000 μM^2 [a]
Unfertilized	8	25 ± 3
Fertilized	11	39 ± 3[b]
Two-cell	10	44 ± 4
Three-cell	1	75[b]

[a] Number of reduced silver grains per 100 μM^2 of embryonic nucleoplasm or ooplasm ± standard error.
[b] Significantly different from previous cell stage ($P < 0{\cdot}05$).

Table 3. [^{3}H]-Leucine incorporation by squirrel monkey oocytes and by embryos produced by *in vitro* fertilization.

Cell stage	*n*	No. of grains/1000 μM^2
Immature oocyte	10	329 ± 20
Mature oocyte	8	164 ± 20[a]
Fertilized	7	118 ± 40
Two-cell	3	220 ± 25

[a] Significantly different from previous cell stage ($P < 0{\cdot}05$).

Table 4. [^{3}H]-Estradiol and [^{3}H]-progesterone uptake by squirrel monkey oocytes and by embryos produced by *in vitro* fertilization.

		Uptake (pmol/embryo/2 h)		
Cell stage	*n*	Estradiol	*n*	Progesterone
Unfertilized	8	0·59 ± 0·07	6	0·21 ± 0·02
Fertilized	5	0·87 ± 0·17	5	0·49 ± 0·05
Two-cell	4	1·20 ± 0·40	4	0·38 ± 0·10

this was not statistically significant. Progesterone uptake increased at *in vitro* fertilization and then remained constant at the two cell stage. The results with 2 DG have been reported elsewhere (Hutz *et al.*, 1984). Briefly summarized, the addition of insulin increased 2-DG accumulation by unfertilized oocytes over controls without insulin, but not to a significant extent (from 13·95 ±2·4 to 15·64 ± 3·78 to 18·84 ± 1·22 fmol/oocyte/3 h) for the control and two levels of insulin respectively. There was no change in 2-DG accumulation at fertilization. These results suggest low utilization of glucose by the early preimplantation primate embryos similar to that demonstrated

for other mammalian species. 2-DG did appear to be a good viability indicator of early primate embryos.

The temporal stages of development for squirrel monkey oocytes in the preliminary stages of development have already been reported (Dukelow *et al*., 1983). Capacitation in this species requires 2–4·7 h (Kuehl and Dukelow, 1982) and by 6 h after fertilization condensation and swelling of the sperm head has occurred. The first pronucleate stage is evident by 10h after fertilization with initial cleavage occurring from 16 to 20h. Subsequent development of the squirrel monkey embryo, compared with the rhesus monkey, cynomolgus monkey, baboon and human are indicated in table 5.

Table 5. Comparative rates of primate preimplantation development.

Species (type of culture)	(Hours after fertilization) Two polar bodies	2 Cell	4 Cell	8 Cell	16 Cell	Morulla	Blastocyst
Squirrel Monkey							
in vivo fert., *in vivo* culture[a]							96
in vitro fert., *in vitro* culture[b]	6–22	20–40	46–52	52–72			
Rhesus Macaque							
in vivo fert., *in vitro* culture[c]		24–36	36–48	48–72	72–96		
in vitro fert. *in vitro* culture[d]		26	38	54	87		
Cynomolgus Macaque							
in vivo fert., *in vitro* culture[e]		24	48	48–72			
Baboon							
in vivo fert., *in vivo* culture[f,g]	24		48	48–72	96–120	120–148	96–144
in vitro fert., *in vitro* culture[h]	6–12	24		72*			
Human							
in vivo fert., *in vivo* culture[i,j,k]		30		72	96	96–120	120
in vitro fert., *in vitro* culture[l]	12	38	38–46	51–62	85	111–135	123–147

* Xenogenously fertilized, a) Ariga and Dukelow, 1977; b) Kuehl and Dukelow, 1979; c) Lewis and Hartman, 1941; d) Bavister *et al*., 1983; e) Kreitmann *et al*., 1982; f)Kraemer and Hendrickx, 197 1; g) Pope *et al*., 1982; h) Kuehl *et al.,* 1984; i) Hertig, 1975; j) Croxatto *et al*., 1972; k) Avendano *et al*., 1975; 1) Edwards and Steptoe, 1975.

Discussion

Based on the recovery and fertilization rate of squirrel monkey oocytes, this animal provides a very useful model for the examination of other facets of abnormality. With

increasing use of *in vitro* fertilization in human infertility cases there remains the problem of genetic safety of *in vitro* maturation and fertilization procedures. In the present studies, hormonal induction of follicular development and *in vitro* fertilization were found not to be major causes of chromosomal abnormality. The levels of missing or extra chromosomes were found to be quite comparable to that reported for other laboratory species. The single measure that was adverse to the *in vitro* fertilization system was the 16·7% incidence of triploidy, probably caused by polyspermy. The exact cause of this polyspermy is unknown at this time, but a similar event has been noted in the *in vitro* fertilization system of other laboratory animals and humans (Lopata *et al*., 1978). Varying numbers of sperm were utilized in the culture system to yield the present data and presently a comprehensive analysis of the effect of sperm number on the incidence of polyspermy is being undertaken.

Autoradiographic analysis of RNA synthetic capacity after *in vitro* fertilization and cleavage showed that uridine uptake is many times lower than similar studies with hamster embryos (Hutz, 1983). Precursor pools may differ appreciably between species. Nevertheless, RNA synthesis did appear to remain quite low in early preimplantation development and this correlates well with the very low RNA synthesis found in the early preimplantation stages of the mouse (Knowland and Graham, 1972). In that species, increases of RNA synthesis are not seen until the eight cell and morula stages and again at the blastocyst stage. Similarly the decrease of the cellular incorporation of leucine with maturation and the low levels at fertilization are similar to that found in other mammalian species (Baker *et al*., 1969; Brinster, 1971; Schultz *et al*., 1978). These low levels of protein synthesis correlate with the low number of polyribosomes found in the morula and blastocyst stages of primate embryos fertilized *in vivo* (Enders and Schlafke, 1981) and *in vitro* (Yorozu *et al*., 1984). Since the size of the precursor pool for leucine is not known for primate ova, absolute measurements of protein synthesis could not be made. Apparent increases in protein synthesis occur late in preimplantation development (at the eight cell and blastocyst stages) in the mouse (Epstein, 1975).

The studies demonstrating uptake of estradiol by primate oocytes after fertilization apparently indicate that steroid receptors are present very early in the development but their functional significance is unknown. Because of the scarcity of available material we have not been able to separate cytosol from nuclear receptors in primate ova. Steroids can effect various aspects of metabolism but do not alter RNA synthetic rates in other laboratory animals. Steroids may rather play a role in effecting membrane changes and therefore cleavage of the early preimplantation embryos (Warner and Tollefson, 1978). Near the time of implantation embryos possess aromatase activity and are able to synthesize steroids (Dickmann and Dey, 1974; Hoversland *et al*., 1982).

These biochemical studies demonstrate normal development of preimplantation primate embryos, similar to that or other mammalian species, after *in vitro* fertilization in culture. Such investigations allow evaluation of the requirements of the embryo for proper *in vitro* development and enhance the success with a nonhuman primate *in vitro* fertilization system. Ethical considerations preclude similar investigations with human embryos. However limited studies of RNA synthesis of unfertilized human oocytes (Hutz *et al*., 1983) indicate similarity between the human and squirrel monkey oocyte.

The stages of temporal development of *in vitro* fertilized squirrel monkey embryos (table 5) readily indicate the similarity between the squirrel monkey, other nonhuman

primates and the human. These studies amply demonstrate the usefulness of the squirrel monkey model.

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