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In vitro fertilization and embryo development in the marmoset monkey (*Callithrix jacchus*)

L. J. Wilton*, V. S. Marshall, E. C. Piercy and H. D. M. Moore†

Institute of Zoology, Regent's Park, London, NW1 4RY, UK

Oocytes aspirated from preovulatory (i.e. ≥ 2 mm) follicles of marmoset monkeys were graded for maturity according to the degree of cumulus expansion, grade I being most mature and grade IV least mature. After preincubation for 2–5, 9–11 or 21–29 h, 82% of oocytes could be fertilized using epididymal spermatozoa and only 2.3% were polyspermic. Fertilization rate was lowest (60%) in grade IV oocytes and all oocytes preincubated for 2–5 h (53%). Fertilization rate increased to 92% in oocytes preincubated for 21–29 h. Embryos developed *in vitro* to a mean of eight cells. Embryo development was unaffected by oocyte maturity but correlated with preincubation time. Oocytes preincubated for 2–5 h developed into embryos with significantly fewer cells than those preincubated for 9–11 or 21–29 h ($P < 0.001$). Fifty-six per cent of embryos showed delayed cleavage and these had fewer cells than non-delayed embryos ($P < 0.001$). When oocytes were preincubated for 2–5 h, development of all resulting embryos was delayed. However, only 17 and 58% of embryos developing from oocytes preincubated for 9–11 and 21–29 h, respectively, were delayed and this was independent of oocyte maturity.

Introduction

In recent years *in vitro* fertilization (IVF) has provided access to human preimplantation embryos. Research studies on human embryos have increased our knowledge of early human development but for ethical reasons an empirical approach has had to be adopted. This precludes detailed investigations over the peri-implantation period so crucial for embryo development. For this reason, we must rely on non-human primate models to investigate pre- and postimplantation development fully. IVF systems have been developed for rhesus monkeys (Bavister *et al.*, 1983; Boatman *et al.*, 1986; Wolf *et al.*, 1989; Morgan *et al.*, 1990), squirrel monkeys (Dukelow *et al.*, 1983), cynomolgus monkeys (Balmaceda *et al.*, 1984), baboons (Irsigler *et al.*, 1984) and chimpanzees (Gould, 1983) with varying degrees of success. In particular, *in vitro* culture of embryos produced by IVF has been difficult in all non-human primate species (see Boatman, 1987). In this laboratory the common marmoset (*Callithrix jacchus*) has been successfully used for IVF and embryo transfer (Summers *et al.*, 1987; Lopata *et al.*, 1988) and a small number of embryos have been cultured *in vitro* (Lopata *et al.*, 1988). In the present study we have set out to improve marmoset IVF by optimizing the timing of follicular aspiration and insemination and have demonstrated significant effects on embryo development *in vitro*. As well as aiding our knowledge of preimplantation primate development, these techniques could also be applied to captive breeding of closely related endangered species such as the golden lion tamarin (*Leontopithecus rosalia*).

*Present address: Centre for Early Human Development, Monash Medical Centre, Clayton, Victoria, Australia 3168.

†Present address: Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2UH, UK.

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Materials and Methods

Animals

Adult female marmoset monkeys (*Callithrix jacchus*) of proven fertility were housed with their male partners. All females in our colony are routinely monitored to determine the day of ovulation by measuring plasma progesterone concentrations using an enzyme-linked immunoassay (Hodges *et al.*, 1988) in 0.3 ml of blood collected from the femoral vein. In the females used in this study, premature luteolysis was induced by an injection of 0.5 μg of cloprostenol, an analogue of prostaglandin $F_{2\alpha}$ (Estrumate: ICI, Macclesfield) at 09:00 h between 13 and 22 days after the previous ovulation (Summers *et al.*, 1985). At 13:00 h on day 7 after cloprostenol treatment, animals were given an injection of 75 iu of human chorionic gonadotrophin (hCG; Chorulon: Intervet Laboratories, Cambridge). A total of 41 ovarian cycles were investigated in 36 marmosets. There was an interval of 4–13 months between investigations in the five animals that were used twice.

Follicular aspiration and oocyte recovery

Oocytes were surgically collected 21–24 h after hCG injection. Animals were anaesthetized and the ovaries and uterus exposed by a mid-line laparotomy. Only follicles greater than 2 mm, and hence defined as preovulatory (Harlow *et al.*, 1988), were aspirated. An aspiration pipette was prepared by pulling a 1.5 mm diameter thin-walled glass capillary (Clark Electromedical, Reading) over a flame and then breaking it at a diameter of 0.7–0.8 mm. Follicular puncture was facilitated by a sharp spike on the pipette that was produced by touching the tip on the

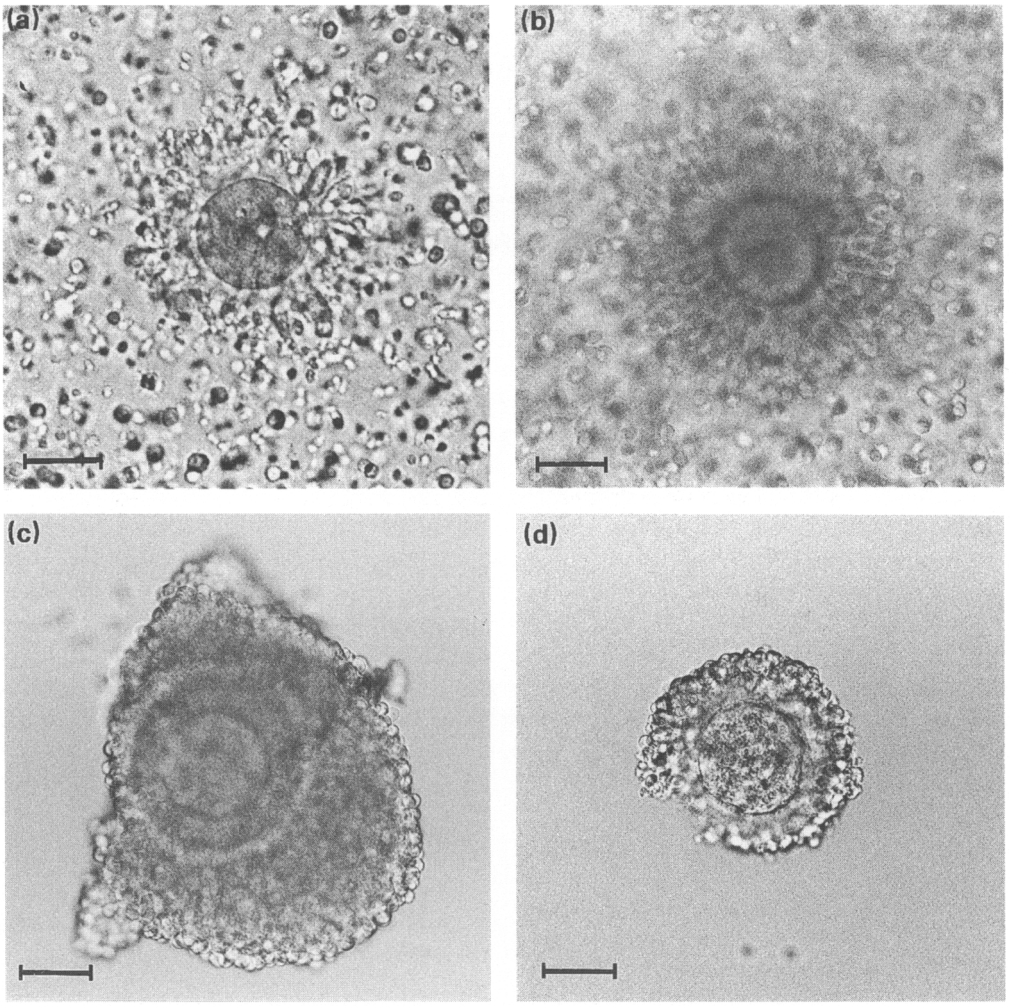

Fig. 1. Marmoset oocytes at different stages of maturation. (a) Grade I – well expanded cumulus cells; (b) grade II – extensive cumulus cells that are not fully expanded; (c) grade III – several layers of non-expanded cumulus cells; (d) grade IV – only a few layers of tightly packed cumulus cells. Bars = 50 μm.

heated glass bead of a microforge. The pipette was attached to a micrometer syringe which enabled controlled suction of the follicle. Follicular contents were expelled into alpha-modified minimum essential medium (MEM; Gibco, Uxbridge) buffered with 25 mmol Hepes l^{-1} and containing 1% heat-inactivated marmoset serum, 5 mg streptomycin sulfate $(100\ ml)^{-1}$, 6 mg penicillin $(100\ ml)^{-1}$ and 1 iu heparin ml^{-1}. It was not possible to visualize extrusion of the first polar body through the cumulus cells and oocytes were therefore scored for maturity according to degree of cumulus expansion. Grade I oocytes had extensive, well-expanded cumulus; grade II oocytes had several layers of cumulus cells that were not fully expanded; grade III oocytes had several layers of densely packed cumulus cells and grade IV oocytes had only 2–3 layers of tightly packed surrounding cells (Fig. 1).

Before insemination, oocytes were preincubated in MEM containing 10 μmol dibutyryl cAMP l^{-1}, 10 μmol caffeine l^{-1}, 5 mg streptomycin sulfate $(100\ ml)^{-1}$, 6 mg penicillin $(100\ mol)^{-1}$ and 10% marmoset serum for 2–5, 9–11 or 21–29 h at 37°C in an atmosphere of 5% CO_2 in air or, in later experiments, 5% CO_2, 5% O_2 and 90% N_2, although this made no difference to the results.

Preparation of spermatozoa

Preliminary work demonstrated that very few spermatozoa collected by electroejaculation could be separated from the seminal secretions that coagulated immediately on collection and resisted all attempts at liquefaction or separation. Moreover, the few spermatozoa that were free swimming were extremely sensitive even to gentle centrifugation and had poor motility and viability, surviving less than 8 h *in vitro*. In contrast, epididymal spermatozoa were highly motile and survived for more than 48 h *in vitro* in media containing marmoset serum. Marmoset spermatozoa whether epididymal or electroejaculated survived for less than 6 h in media supplemented with fetal calf serum (data not shown). Consequently epididymal spermatozoa were used for all the experiments described here.

The epididymides were carefully dissected from male marmosets of proven fertility and coarsely minced in MEM

containing dibutyryl cAMP, caffeine, antibiotics and 10% marmoset serum as for oocyte preincubation.

In vitro *fertilization and embryo culture*

Spermatozoa were diluted to a concentration of between 5 and 10 × 10^5 ml^{-1} and oocytes added for 6–8 h. In some experiments, immature oocytes were inseminated for 12–22 h. Oocytes were then washed and incubated in MEM plus 10% marmoset serum and insulin–transferrin–sodium selenium supplement (Lopata *et al.*, 1988) for 4–20 h after which time in most cases the cumulus had migrated away from the zona pellucida. If this failed to occur, oocytes were gently aspirated through a flame polished pipette to dislodge the cumulus cells.

Fertilization was confirmed by visualizing the clearly distinguishable pronuclei using an inverted microscope fitted with differential interference contrast optics. In most cases two polar bodies could also be identified. Embryos were cultured in MEM supplemented with 10% marmoset serum at 37°C in 5% CO_2 in air or, in later experiments, in a mixture of 5% CO_2, 5% O_2 and 90% N_2, although this made no difference to the results. Embryos were considered to have arrested if no cells had cleaved within the previous 48 h.

Statistical analyses

Fertilization rates were compared using a χ^2 or V^2 (when sample size was less than 10) test. Student's *t* tests were used to compare mean cell number of embryos. The level of significance was $P < 0.05$.

Results

Follicular development was successfully controlled so that in 40 of 41 surgical procedures, pre-ovulatory follicles (i.e. > 2 mm) were present and no animal had ovulated at the time of laparotomy. In the 41 laparotomies, there were 110 pre-ovulatory follicles, a mean of 2.7 follicles per animal. All follicles were aspirated and 88 of 110 (80%) oocytes were recovered overall. However, in the 26 most recent laparotomies 62 of 67 (93%) of oocytes were recovered. Of the 88 oocytes recovered 19 (22%), 36 (41%), 19 (22%), 13 (15%), and 1 (1%) were grade I, II, III, IV and atretic, respectively.

One grade III, three grade IV and the atretic oocyte were not inseminated. Eighty-three oocytes were inseminated and 68 (82%) fertilized. Two (2.3%) of these were polyspermic, confirmed by the presence of three pronuclei. Fertilization rate was dependent on oocyte maturity. Grade IV oocytes had a fertilization rate of 60%, whereas 78–92% of grade I–III oocytes fertilized (Table 1). Fertilization rate also depended upon the duration of oocyte incubation before insemination (preincubation). As shown in Table 1, oocytes preincubated for 2–5 h had a fertilization rate of 53% (10 of 19). This increased to 86% (12 of 14, $P < 0.002$) and remained high at 92% (46 of 50) after 9–11 and 21–29 h oocyte preincubation, respectively. The effect of longer preincubation of oocytes on fertilization rate was most marked in least mature oocytes. Although the numbers in these groups were small, fertilization appeared to be improved with longer preincubation. Only one out of three grade IV oocytes incubated for 2–5 or 9–11 h fertilized. However, 5 of 7 (71%) grade IV oocytes fertilized after 21–29 h preincubation (Table 1). Similarly, the fertilization rate of grade III oocytes increased from 25 to 67 and 100% ($P < 0.002$) by increasing the preincubation time from 2–5 h to 9–11 and 21–29 h, respectively (Table 1). The fertilization rate of grade I and II oocytes increased to 100% after 9–11 h preincubation and remained high when oocytes were preincubated for 21–29 h ($P < 0.025$).

Table 1. Fertilization rate (%) of marmoset oocytes of grades I–IV after preincubation for 2–5, 9–11 or 21–29 h

Oocyte grade	Duration of preincubation (h) 2–5	9–11	21–29	Total
I	2/5 (40)	2/2 (100)	11/12* (92)[a]	15/19 (79)
II	7/9 (78)	7/7 (100)	19/20* (95)	33/36 (92)
III	1/4 (25)	2/3 (67)	11/11 (100)[b]	14/18 (78)
IV	0/1 (0)	1/2 (50)	5/7 (71)	6/10 (60)
Total	10/19 (53)	12/14 (86)[c]	46/50 (92)[c]	68/83 (82)

*Includes one polyspermic fertilization.
[a]$P < 0.025$ compared with grade I oocytes preincubated for 2–5 h.
[b]$P < 0.002$ compared with grade III oocytes preincubated for 2–5 h.
[c]$P < 0.002$ compared with all oocytes preincubated for 2–5 h.

Of the 66 normally fertilized embryos, 60 (91%) cleaved to two cells. One embryo was inadvertently lost at the two-cell stage. Fifty-three (82%) of the remaining 65 embryos reached the four-cell stage and 31 (48%) divided to eight cells. Of these, seven (11%) cleaved to 16 cells and began to compact. Only one embryo reached the 32-cell stage. Overall, the mean (± SEM) maximum cell number reached for normally fertilized embryos was 7.7 ± 0.7 (Table 2). The two triploid embryos divided to six and 12 cells. When data from all preincubation times were combined, there was no significant difference in the mean maximum cell number between the embryos produced from different grade oocytes (Table 2).

However, increasing the duration of oocyte preincubation had a beneficial effect on embryo development. Forty per cent (4 of 10) of oocytes preincubated for only 2–5 h failed to cleave and none developed further than six cells (Fig. 2). However, only 2 of 52 (3.8%) of oocytes preincubated for 9–11 or 21–29 h failed to cleave and a total of 29 of 52 (56%) of these oocytes reached at least the eight-cell stage (Fig. 2). This was also reflected in the mean maximum cell number. Oocytes preincubated for 2–5 h developed into embryos with mean maximum cell number of 2.8 ± 0.7 and oocytes preincubated for 9–11 and 21–29 h cleaved to a mean maximum cell number of 9.6 ± 2.5 ($P < 0.001$) and 8.3 ± 0.6 ($P < 0.001$), respectively (Table 2 and Fig. 2). This effect was most marked in grade II oocytes. The mean maximum cell number significantly increased from 2.3 ± 0.6 to 13.7 ± 3.4 ($P < 0.001$) after grade II oocytes were preincubated for 9–11 h and to 9.4 ± 1.0 ($P < 0.001$) after 21–29 h preincubation.

Marmoset embryos should reach the four-cell stage on day 2 after insemination (Harlow, 1984). In the present study, 35 of

Table 2. Mean ± SEM maximum cell number of marmoset embryos developing from grades I–IV oocytes preincubated for 2–5, 9–11 or 21–29 h*

Oocyte grade	Length of preincubation (h) 2–5	9–11	21–29	Total
I	4, 6 ($n = 2$)	4, 5 ($n = 2$)	7.5 ± 1.4 ($n = 8$)	6.6 ± 1.0 ($n = 12$)
II	2.3 ± 0.6 ($n = 7$)	13.7 ± 3.4[b] ($n = 7$)	9.3 ± 1.0[b] ($n = 18$)	8.8 ± 1.2 ($n = 32$)
III	1 ($n = 1$)	1, 8 ($n = 2$)	7.6 ± 1.1 ($n = 10$)	6.6 ± 1.1 ($n = 13$)
IV	–	1 ($n = 1$)	6.5 ± 1.7 ($n = 4$)	5.4 ± 1.7 ($n = 5$)
Total	2.8 ± 0.6 ($n = 10$)	9.6 ± 2.5[a] ($n = 12$)	8.3 ± 0.6[a] ($n = 40$)	7.7 ± 0.7 ($n = 62$)

*Where there were fewer than three embryos per group the maximum cell number of each embryo is shown, statistical comparisons were not performed when $n < 3$.
[a] $P < 0.001$ compared with 2–5 h preincubation (total).
[b] $P < 0.001$ compared with 2–5 h preincubation (grade II only).

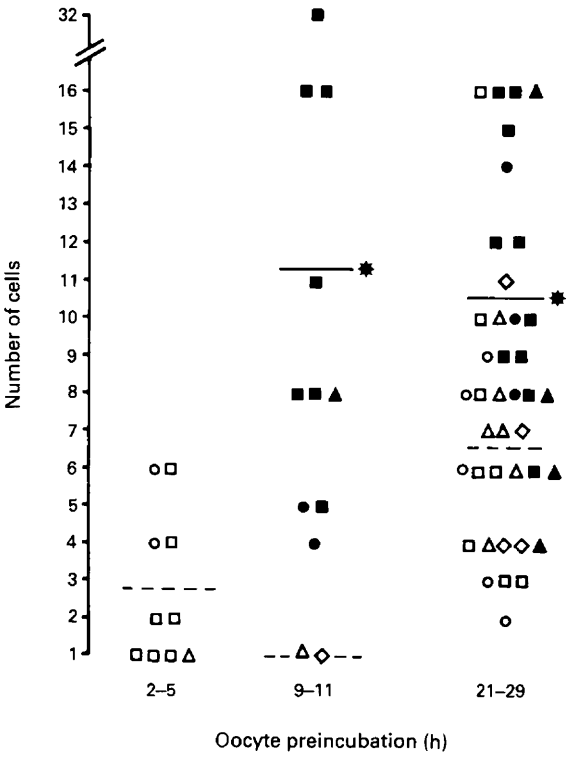

Fig. 2. Maximum cell number reached by each embryo according to oocyte grade and preincubation time. Non-delayed embryos (closed symbols); delayed embryos (open symbols); embryos developing from grade I (●, ○), grade II (■, □), grade III (▲, △) and grade IV (◆, ◇) oocytes; mean cell number of delayed (– – –) and non-delayed (——) embryos. *$P < 0.001$ compared with delayed embryos.

62 (56%) embryos were delayed on day 2 (Fig. 2). Delayed embryos reached a mean maximum cell number of 5.2 ± 0.6, which was significantly less than 10.8 ± 1.1 ($P < 0.001$) cells in embryos that were not delayed (Fig. 2). All ten embryos developing from oocytes preincubated for only 2–5 h were delayed. Only 2 of 12 (17%) oocytes preincubated for 9–11 h were delayed; however, 23 of 40 (58%) embryos from oocytes that were preincubated for 21–29 h were delayed. When considering only embryos developing from oocytes preincubated for 21–29 h, embryos that were not delayed on day 2 reached a significantly higher mean maximum cell number than delayed embryos (10.5 ± 0.9 versus 6.6 ± 0.7, $P < 0.005$). This difference was independent of the maturity of the oocyte from which the embryo had developed. That is, the mean maximum cell number of delayed embryos was not significantly different whether the embryos had developed from grade I, II, III or IV oocytes (data not shown). This was also true for embryos that were not delayed (data not shown).

Discussion

The treatment protocol used to control the ovarian cycle and induce follicular maturation was highly successful. On all except one occasion follicles greater than 2 mm were present and were aspirated. No animals had ovulated at the time of laparotomy and the fertilization rate was high. This protocol is a modification of that reported by Lopata *et al.* (1988). They found that if cloprostenol was administered at 09:00 h on day 0 and hCG at 09:00 h on day 7, oocytes could be collected at laparotomy 24 h later but 67% were polyspermic after fertilization. If instead they administered hCG at 17:00 h on day 7, the polyspermy rate decreased but two of 14 animals had already ovulated at laparotomy. This indicates that the timing of hCG administration relative to cloprostenol is critical to the success of oocyte collection and normal fertilization. We have given hCG exactly halfway between the two times used by Lopata *et al.* (1988) and found that no animals had ovulated, the fertilization rate was high and the polyspermy rate very low.

It has previously been demonstrated that, in marmosets, most oocytes from follicles smaller than 2.5 mm are immature and cannot be fertilized normally (Lopata *et al.*, 1988). Hence we only aspirated follicles that were greater than 2 mm and were able to recover an average of 2.1 oocytes per animal. Obviously, it would be advantageous to increase the number of oocytes produced by each animal. However, preliminary studies to superovulate the marmoset ovary with human menopausal gonadotrophin or clomiphene citrate have been unsuccessful (Harlow, 1984): there was no increase in the number of developing follicles. Moreover, other non-human primates develop antibodies to gonadotrophin preparations and become resistant to superovulation (Platia *et al.*, 1984; Bavister *et al.*, 1986; Dukelow and Vengesa, 1986; Rotten *et al.*, 1986; Cranfield *et al.*, 1989). Marmosets do not develop antibodies to hCG and can be used repeatedly for collection of pre-ovulatory oocytes.

Lopata *et al.* (1988) found that 61% of marmoset oocytes collected from follicles greater than 2.5 mm fertilized *in vitro*. In the present study, we have achieved a higher fertilization rate, 82%, for all oocytes. Moreover, to our knowledge, we have achieved the highest consistent fertilization rate of any

non-human primate species. In other species, fertilization rates *in vitro* range from 39% in the baboon (Fourie *et al.*, 1987) to 71% in the cynomolgus macaque (Fujisaki *et al.*, 1989). As expected, the fertilization rate was higher in more mature oocytes. However, when cultured for up to 29 h *in vitro*, grade III and IV oocytes continued to mature, as demonstrated by expansion of the cumulus cells. This *in vitro* maturation also increased the fertilization rate up to 100% in grade III oocytes. This demonstrates that nuclear maturation of marmoset oocytes will occur in culture. The fertilization rate of grade I and II oocytes was 100% after only 9–11 h preincubation and was not compromised by extended *in vitro* maturation for 21–29 h.

Although a very high proportion of fertilized oocytes cleaved, the development of marmoset embryos *in vitro* was poor. On average, embryos developed to a maximum of 7–8 cells and only 11% reached 16 cells. In the only other published study of culture of *in vitro* fertilized marmoset embryos, only a small number of embryos were examined and 40% developed to the morula stage and 20% to the blastocyst stage using conditions and media similar to those described here (Lopata *et al.*, 1988). The aim of our study was to culture *in vitro* fertilized marmoset embryos to advanced blastocyst stages and we have yet to determine whether embryos produced in our IVF system can develop into viable offspring. However, it has been demonstrated that *in vitro* fertilized marmoset embryos can develop *in vivo* and, when transferred to recipients at the four- to six-cell stage, 60% result in live births (Lopata *et al.*, 1988). Preimplantation embryos of other non-human primates have also proved very difficult to culture *in vitro* (Boatman, 1987; Wolf *et al.*, 1990; Morgan *et al.*, 1991) with little development past the eight- to 16-cell stage (Lanzendorf *et al.*, 1990).

We have found that the maximum cell number reached by marmoset embryos *in vitro* did not depend on initial oocyte maturity provided that the oocytes were preincubated for sufficient time. Thus, after 21–29 h preincubation, there was no difference in the mean maximum cell number between embryos derived from grade I and grade IV oocytes.

Oocyte preincubation time had a significant effect on embryo development. Embryos derived from oocytes preincubated for only 2–5 h developed to a mean maximum of only three cells, whereas oocytes preincubated for 9–11 h developed to an average of ten cells. There was no further advantage in preincubating oocytes for longer periods. This would suggest that although oocytes can attain nuclear maturity by longer preincubation times as demonstrated by the increase in fertilization rate, there is a limit to the extent of cytoplasmic maturity that can be achieved during *in vitro* culture. This is also true of rhesus oocytes (Boatman, 1987; Lanzendorf *et al.*, 1990; Morgan *et al.*, 1991).

Because the embryos in the present study developed poorly, we have been unable to ascribe cell cycle times and cleavage rates accurately. The only comparable data come from naturally fertilized embryos, flushed from the uterus, which suggests that marmoset embryos reach the four-cell stage on day 2 but after this developmental rates may vary. Morulae can be collected between days 5 and 7 and blastocysts between days 7 and 9 after ovulation (Harlow, 1984); hence comparisons with *in vitro* development particularly during later preimplantation stages must be made with caution. Some marmoset embryos in the present study continued to cleave slowly for up to 12 days. It is hoped that optimization of the *in vitro* culture of marmoset embryos will enable further characterization of their early development. However, we have been able to show that embryos that reached the four-cell stage at the expected time (i.e. day 2) developed significantly further than those that were retarded, suggesting that extended preincubation of oocytes has a detrimental effect on cytoplasmic maturity. We found that 83% of embryos derived from oocytes preincubated for 9–11 h reached the four-cell stage at the expected time but that only 43% of embryos derived from oocytes preincubated for 21–29 h were cleaving at the normal rate at the four-cell stage. Prolonged preincubation with resultant ageing of the oocytes may result in the inability of the cytoplasm to support normal cleavage. If true, it might be expected that the least mature oocytes would be affected to a lesser extent but this was not the case. Delayed cleavage, caused by prolonged oocyte preincubation, occurred to a similar extent in embryos derived from all four grades of oocyte. Another explanation is that the *in vitro* environment is supportive of oocyte maturation for approximately 10 h but after longer periods has an adverse effect on cytoplasmic factors that are responsible for early cleavage.

In all the experiments described here, we have used MEM supplemented with 10% serum as a culture medium, as this has previously been demonstrated to support marmoset preimplantation development *in vitro*, albeit using only a small number of embryos (Lopata *et al.*, 1988). In macaques, simple salt media such as modified Tyrode's solutions supplemented with bovine serum albumin support embryo development during early cleavage stages but not morula formation (Boatman, 1987). Macaque embryos develop from the two-cell stage to at least the morula stage in a more complex media (Boatman, 1987) so we avoided, at least in our initial studies, culturing early cleavage stage marmoset embryos in simple media. However, having established an optimal system for marmoset IVF, we will now test alternative embryo culture techniques including different media and co-culture on oviduct and uterine epithelia.

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