Aust. J. Biol. Sci., 1987, **40**, 389–95

Effects of Coculture with Uterine Epithelial Cells on the Metabolism of Glucose by Mouse Morulae and Early Blastocysts

N. K. Khurana and R. G. Wales

School of Veterinary Studies, Murdoch University, Murdoch, W.A. 6150

Abstract

Coculture of mouse morulae/early blastocysts with isolated endometrial epithelial cells reduced incorporation of glucose carbon into embryonic glycogen but had no significant effect on incorporation into other internal carbon pools during a 5-h culture in serum-supplemented Dulbecco's modification of Eagle's minimum essential medium. Turnover of glycogen pools during 24-h chase culture of pulse-labelled embryos was unaffected by the presence of uterine epithelial cells recovered from day-4 pregnant or non-pregnant mice. However, significantly more label was retained in non-glycogen macromolecules during chase in the presence of endometrium recovered from non-pregnant than from pregnant uteri.

Introduction

Development of the preimplantation mammalian embryo *in vivo* occurs in a complex luminal environment whose volume and composition is under the control of the ovarian hormones progesterone and oestradiol (Tantayaporn *et al.* 1974; Surani 1977). Furthermore, as the volume of fluid in the uterus is small (Hoversland and Weitlauf 1981; Edirisinghe 1982), there is relatively close apposition between the embryo and the cells of the endometrial lining, leading to the possibility of a direct influence of the latter cells on embryonic development.

Qualitative and quantitative differences exist between embryos cultured *in vitro* and those developing *in utero*, suggesting an active involvement of the uterine environment. One influence of the uterine environment on the embryo is the decreased accumulation of glycogen *in vivo* compared to *in vitro* (Ozias and Stern 1973). This difference is due to the increased turnover of the polymer *in utero* (Edirisinghe *et al.* 1984) and is controlled by maternal progesterone (Edirisinghe and Wales 1984) but does not appear to be due to a direct action of steroids on embryos (Khurana and Wales 1987*a*).

Ovarian steroids could influence embryo metabolism through their effect on the endometrium. Potter *et al.* (1981) noted similarities between *in vitro* and *in vivo* responses of mouse uterine epithelium to ovarian hormones and suggested that the epithelium was the target for steroid induced changes in the uterus. Thus a system of coculture involving embryos and isolated endometrial cells in the presence of ovarian steroids could be a useful model for studying the cell-to-cell interaction between embryo and endometrium.

The present study was undertaken to investigate this interaction on the incorporation and turnover of glucose carbon in the embryo. As culture of endometrial cells requires a more complex medium than that normally used for embryo culture, experiments were also performed to investigate the effects of such media on development and substrate turnover by the embryos.

0004-9417/87/040389$02.00

Materials and Methods

General

Morulae and early blastocysts were collected on the fourth day of pregnancy from random bred Swiss mice of the Quackenbush (Q(s)) strain as described previously (Pike and Wales 1982) using a modified Krebs–Ringer–bicarbonate solution containing 25 mM lactate, 0.25 mM pyruvate and supplemented with antibiotics and 1 mg/ml bovine serum albumin as the basic medium (Brinster 1965). For each replicate of an experiment, approximately equal numbers of embryos (36–50) were assigned randomly to each treatment group from the pool of embryos collected. All incubations were carried out in an incubator at 37°C and in a humidified atmosphere of 5% CO_2–95% air. Where treatments involved coculture of embryos with isolated endometrial cells, Dulbecco's modification of Eagle's minimum essential medium supplemented with 10% foetal calf serum (serum-supplemented DMEM) was used in order to preserve the viability of the latter cells during the period of the experiment. The concentration and specific radioactivity of glucose in this medium were varied according to the needs of the particular experiment.

The DMEM was prepared from powdered constituents (Flow Laboratories Inc., Sydney) and was reconstituted in triple distilled water after adding sodium bicarbonate. The pH was adjusted to 7.2, penicillin and streptomycin were added, and sterilization was carried out by membrane filtration (0.22 μm). The reconstituted medium was stored at 4°C for up to 2 weeks in tightly capped bottles gassed with 5% CO_2 in air. Glutamine and foetal calf serum (10% v/v) were added fresh on the day of use. Where progesterone was included in media, it was added as described previously (Khurana and Wales 1987*a*).

Isolation of Uterine Epithelium

Uterine epithelium was isolated enzymatically as described previously (Sherman 1978). Briefly, uteri were cleaned and trimmed in calcium-and magnesium-free phosphate-buffered saline (PBS, solution 'A' of Dulbecco and Vogt 1954) and slit along their lengths. They were then placed in an ice-cold solution containing 0.5% (w/v) trypsin (bovine pancreatic, type III, Sigma Chemical Co., St Louis, MO, U.S.A.) and 2.5% (w/v) pancreatin (Sigma Chemical Co.) in Hank's balanced salt solution but calcium- and magnesium-free. After 2 h at 4°C, the uteri were incubated 40 min at room temperature and transferred to PBS and the transparent sheets of epithelium were freed with the help of watchmaker's forceps. After rinsing in tissue culture medium, the endometrial epithelial cells were transferred to 6 ml glass culture tubes containing 0.7 ml DMEM. Uterine epithelia were isolated on day 4 after mating to vasectomized or entire males and the material from two animals was used for each replicate.

For confirmation of the cell type isolated, representative specimens of the isolated cells were fixed in 10% (v/v) formal saline, processed histologically and examined under a light microscope. Cell numbers in isolates were estimated by counting on a haemocytometer following trypsinization of isolated cell plaques (Paul 1975). On average, $4 \times 10^5 - 5 \times 10^5$ live cells were used per incubation. Viability of cell plaques immediately after isolation and following culture was assessed by the trypan blue dye exclusion method as recently described by Riehl *et al.* (1983).

Incorporation Studies

To study the effect of coculture with uterine endometrium on incorporation of glucose carbon, mouse morulae/early blastocysts were incubated 5 h in 0.7 ml serum-supplemented DMEM containing 1.5×10^{-6} M progesterone and either with or without the addition of uterine epithelial cells. For this experiment, foetal calf serum was added to glucose-free DMEM and the glucose concentration of the medium was adjusted to 1 mM with [U-^{14}C]glucose (the Radiochemical Centre, Amersham, England) taking into account the glucose content of the added serum. This concentration, rather than the normal concentration of glucose in DMEM (25 mM), was used in order to maintain a high specific activity of the substrate and thus to ensure sufficient labelling of the intracellular carbon pools for accurate radioassay following fractionation. The final specific activity of the [U-^{14}C]glucose in the medium was 111 kBq/μmol. Epithelial cells recovered from both pregnant and non-pregnant uteri were tested and in each case duplicate tubes for each treatment were included.

Turnover Studies

Embryos were pulse labelled for 2 h in droplets of Ringer-based basic medium under oil, the medium being supplemented with 0.28 mM [U-^{14}C]glucose (specific activity 1.11 MBq/μmol). Following pulse culture, the embryos were washed through two changes of non-radioactive medium. A sample of pulsed embryos was stored for extraction and the remaining embryos were randomly assigned to duplicate tubes in each treatment group for chase culture using 0.7 ml serum-supplemented DMEM containing 1.5×10^{-6} M

progesterone and 25 mM non-radioactive glucose, with and without the addition of epithelial cells obtained from pregnant and pseudopregnant uteri.

Fractionation of Embryos

At the completion of all experiments, embryos were recovered, washed twice in non-radioactive medium and stored at -70°C for not more than a week prior to fractionation. Samples were fractionated into four biochemical components (acid-soluble glycogen, non-glycogen acid-soluble fraction, acid-insoluble glycogen and non-glycogen acid-insoluble fraction) using the extraction procedure described previously (Edirisinghe *et al.* 1984). The radioactivity in each fraction was determined and the pg-atoms of glucose carbon incorporated into each biochemical fraction was calculated from the radioactivity in the samples and the specific activity of the parent substrate.

Statistical Analysis

The statistical significance was tested by analyses of variance after logarithmic transformation of the data to equalize variances.

Results

Cell Viability and Embryo Development in Coculture

Histological examination by light microscopy confirmed that the cells isolated by enzymic digestion consisted of a single layer of uterine epithelium with no apparent contamination by stromal cells. The viability index of the epithelial cells, as assessed by the trypan blue dye exclusion method, was 94 ± 1.0% (n = 4) immediately after isolation and 91 ± 2.4% (n = 3) and 78 ± 1.8% (n = 3) following incubation in serum-supplemented DMEM for 5 and 24 h, respectively. By contrast, incubation of epithelial cells for 24 h in the simple Ringer-based medium supplemented with serum gave a viability index of 20–30%.

Throughout the course of these experiments, the embryos developed equally well, if not better, in the complex medium and there was a definite trend towards enhanced development in cultures supplemented with uterine epithelial cells when more than 95% of embryos reached the expanded blastocyst stage and many had hatched during the 24-h incubation.

Incorporation Studies

Before using the complex medium for coculture experiments, a preliminary experiment was performed to evaluate the effects of this medium on incorporation of glucose carbon by embryos as compared to incorporation from the single Ringer-based medium used normally for culture of mouse embryos. Both media contained 1 mM glucose. As compared to simple medium, the use of serum-supplemented DMEM reduced the amount of glucose carbon incorporated into the non-glycogen acid-insoluble fraction by approximately 50% (t_2 = 3.02, $P < 0.05$). No other significant effects were observed.

The data for experiments testing the effects of coculture with uterine epithelial cells on incoporation of glucose carbon by embryos are presented in Table 1. The presence of epithelial cells obtained from both pregnant and non-pregnant uteri significantly reduced incoporation into the two glycogen fractions, but had no effect on the non-glycogen fractions.

Turnover Studies

Preliminary trials were conducted to compare the turnover of glucose carbon when serum-supplemented DMEM rather than Ringer-based medium was used for 24-h chase of pulse-labelled embryos. In these trials, glucose concentration in both chase media was 25 mM. This high concentration was required for extended coculture as viability tests showed that survival of the epithelial cells over 24 h was poor in serum-supplemented DMEM containing 1 mM glucose (41%) compared to that in the same mdium containing 25 mM glucose (78%). The results of three replicates of the experiment showed that change to complex media resulted in 35% more label being retained in the acid-soluble glycogen fraction during chase. There

was a similar pattern of degradation in the other three fractions isolated regardless of the medium employed during chase.

The results of the experiments to study the turnover of glucose carbon by pulse-labelled embryos in cultures supplemented with endometrial epithelial cells are summarized in Table 2. During 24-h chase culture, the amount of glucose carbon in all the fractions was significantly depleted. Altering the conditions during chase culture had no effect on the amount of glucose carbon retained in either of the acid-soluble fractions or in acid-insoluble glycogen. However, the amount of label retained in the non-glycogen acid-insoluble pool was significantly greater when the chase took place in the presence of cells from non-pregnant uteri. No such effect was seen when cells from pregnant uteri were used.

Table 1. Incorporation of glucose carbon by mouse morulae–early blastocysts during 5 h pulse culture in serum-supplemented DMEM containing 1.5 × 10^{-6} M progesterone, 1.0 mM glucose and in the presence or absence of endometrial epithelial cells obtained from non-pregnant (NP) or pregnant (P) uteri

Values are means ± s.e.m. of duplicate observations for three replicates

Presence and source of endometrium	Glucose carbon (pg atoms/embryo) incorporated into: Acid-soluble fractions		Acid-insoluble fractions	
	Glycogen	Non-glycogen	Glycogen	Non-glycogen
–	24.48 ± 1.46	6.52 ± 0.64	4.83 ± 0.49	4.24 ± 0.72
+ (P)	14.09 ± 2.04	8.52 ± 2.42	3.00 ± 0.50	3.73 ± 0.72
+ (NP)	18.55 ± 3.18	7.76 ± 1.33	3.54 ± 0.59	4.62 ± 1.10

Summary of the Analyses of Variance (logarithmic transformation). $*P < 0.05$. $**P < 0.01$.

Source of variation	d.f.	Variance ratios: Acid-soluble fractions		Acid-insoluble fractions	
		Glycogen	Non-glycogen	Glycogen	Non-glycogen
Effects of endometrium (P)	1	19.42**	1.00	45.56**	2.25
(NP)	1	6.58*	0.00	17.61**	4.42
Between replicates	2	9.07**	16.06**	40.76**	61.33**
Replicate interactions	4	1.33	6.89**	1.83	1.49
Between duplicates (error)	9	0.0104	0.0085	0.0034	0.0045

Discussion

This study demonstrates that the method of enzymatic splitting yields viable uterine epithelial cells with little evidence of contamination with underlying stromal tissue. Furthermore, these cells can be maintained for at least 24 h in serum-supplemented tissue culture medium and their viability after culture compares favourably with that reported elsewhere (Riehl *et al.* 1983; Salamonsen *et al.* 1985). Thus, coculture of embryos with such isolated cells enables a study of the interactions between embryos and uterine epithelial cells in an environment free from the complications of systemic maternal influences.

The presence of epithelial cells did not influence the turnover of acid-soluble glycogen in pulse-labelled embryos, suggesting that the increased degradation of acid-soluble glycogen *in vivo* (Edirisinghe *et al.* 1984) is not the result of interactions between embryos and the uterine epithelium. Furthermore, the slight reduction in incorporation of glucose carbon into both glycogen fractions on coculture with endometrial epithelial cells is unlikely to explain the discrepancy in glycogen content between cultured and uterine day-5 embryos (Ozias and stern 1973).

Significantly more label was retained in the non-glycogen acid-insoluble fraction during chase culture with uterine epithelial cells from non-pregnant than from pregnant animals. This fraction contains the majority of the non-glycogen macromolecules of the embryo, and thus

there would appear to be an anabolic effect of non-pregnant but not of pregnant endometrial epithelial cells at this time. Since prostaglandins are reported to have an anabolic effect on embryo metabolism *in vitro* (Uehara *et al.* 1984; Khurana and Wales 1987*b*), one possible explanation for the above finding could be the greater synthesis of $PGF_{2\alpha}$ by cells from non-pregnant uteri than cells from pregnant uteri (Maule-Walker and Poyser 1974). The recent finding that there are differences in synthesis and secretion of proteins by isolated epithelial endometrial cells recovered from pregnant and non-pregnant animals (Salamonsen *et al.* 1986) could provide another basis for differential behaviour of the two types of cells.

Table 2. Incorporation of glucose carbon by morulae–early blastocysts during 2 h pulse culture and its turnover during 24 h chase in serum-supplemented DMEM containing 1.5 × 10^{-6} M progesterone, 25 mM glucose and in the presence or absence of endometrial epithelial cells derived from non-pregnant (NP) or pregnant (P) uteri

Values are means ± s.e.m. for four replicates, single observations for pulse culture and duplicate observations for chase treatments

Culture conditions	Presence and source of endometrium	Labelled glucose carbon (pg atoms/embryo) present in: Acid-soluble fractions		Acid-insoluble fractions	
		Glycogen	Non-glycogen	Glycogen	Non-glycogen
Pulse	–	7.14 ± 0.75	5.94 ± 0.99	3.40 ± 0.29	2.71 ± 0.30
Chase	–	6.03 ± 0.51	1.03 ± 0.08	0.92 ± 0.07	1.92 ± 0.21
	+ (P)	5.47 ± 0.57	0.93 ± 0.16	0.97 ± 0.08	1.99 ± 0.24
	+ (NP)	6.51 ± 0.46	1.19 ± 0.20	1.06 ± 0.08	2.30 ± 0.30

Summary of the Analyses of Variance (logarithmic transformation). $*P < 0.05$. $**P < 0.01$

Source of variation	d.f.	Variance ratios: Acid-soluble fractions		Acid-insoluble fractions	
		Glycogen	Non-glycogen	Glycogen	Non-glycogen
Pulse *v.* chase	1	6.18*	200.35**	495.81**	68.47**
With chase					
Effect of endometrium (P)	1	1.16	0.04	0.56	0.36
(NP)	1	0.97	0.23	3.83	13.95**
Between replicates	3	6.20**	8.71**	16.07**	103.39**
Replicate interactions	9	1.76	3.91*	1.22	1.95
Between duplicates	12	0.0066	0.0169	0.0036	0.0015

The results of these coculture experiments also suggest that the coculture of mouse embryos with uterine epithelial cells provides a superior environment for development of embryos. The balance of evidence in the literature suggests that this could be a non-specific effect of coculture rather than a specific effect of endometrial cells *per se*. Several workers have noted increased viability of certain cells cultured in the presence of a feeder layer of another cell type (Kohler and Milstein 1975; Martin 1981). Cole and Paul (1965) were the first to report the enhanced *in vitro* development of mouse embryos cocultured with irradiated HeLa cells. Kuzan and Wright (1982) tested the effects of various cellular and non-cellular substrata on the development of bovine morulae *in vitro* and found that coculture of embryos with bovine fibroblasts promoted embryo hatching and attachment. In another study, Allen and Wright (1984) cocultured porcine embryos with endometrial cells and ovarian fibroblasts and reported that cell-to-embryo contact enhanced the *in vitro* development of embryos with no differences between the two cell types tested. In a recent study, Wiley *et al.* (1986) found that development of mouse embryos was superior when cultured in larger groups and argued that this could result from (unknown) beneficial effects of contact with other cells as happens *in vivo*.

In the preliminary studies, the use of a complex rather than the basic medium for pulse had no effect on glycogen metabolism but led to a significant decrease in the accumulation of glucose carbon in the non-glycogen acid-insoluble fraction. Components of this macromolecular fraction include protein, RNA, DNA and lipid. The suppression of incorporation of glucose carbon into this fraction probably arises as a result of the amino acids and other precursors in the complex medium competing with glucose for entry to the metabolic pathways leading to the synthesis of these macromolecules. In an earlier study, Edirisinghe and Wales (1985) found that the addition of amino acids to the simple Ringer-based medium used in the present study also caused a significant reduction in the incorporation of glucose carbon into this fraction. When a complex medium was used for chase culture, glycogen turnover was reduced, resulting in more label being retained in the acid-soluble glycogen fraction. The preferential use of alternative sources of energy available in complex media could be one possible explanation for this effect. These findings thus suggest that a more complete medium *per se* is unlikely to be a factor in the increased degradation of glycogen *in utero* (Edirisinghe *et al.* 1984).

Although some aspects of the metabolism of glucose by embryos were influenced by the treatments imposed in the present study, the question of the discrepancy in glycogen content of freshly collected and *in vitro* cultured blastocysts (Ozias and Stern 1973) remains unexplained. Further investigations are in progress to study the effects of specific uterine factors (Surani 1977) and of intrauterine oxygen tension (Mitchell and Yochim 1968) on the degradation of embryonic glycogen stores.

Acknowledgments

The technical assistance of Mrs L. McLeod and Ms S. Baker is gratefully acknowledged. The work was supported by a grant from the Australian Research Grants Scheme. One of us (N.K.K.) was supported by a Murdoch University Post-graduate Studentship.

References

Allen, R. L., and Wright, R. W. Jr. (1984). *In vitro* development of porcine embryos in co-culture with endometrial cell monolayers or culture supernatants. *J. Anim. Sci.* **59**, 1657-61.

Brinster, R. L. (1965). Studies on the development of mouse embryos *in vitro*. IV. Interaction of energy sources. *J. Reprod. Fert.* **10**, 227-40.

Cole, R. J., and Paul, J. (1965). Properties of cultured preimplantation mouse and rabbit embryos, and cell strains derived from them. In 'Preimplantation Stages of Pregnancy'. (Eds G. E. W. Wolstenholme and M. O'Conner.) pp 82-112. (Little Brown & Co.: Boston.)

Dulbecco, R., and Vogt, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **99**, 167-82.

Edirisinghe, W. R. (1982). Studies on the metabolism of glucose by the preimplantation mouse embryo. Ph.D. Thesis, Murdoch University.

Edirisinghe, W. R., and Wales, R. G. (1984). Effect of parental administration of oestrogen and progesterone on the glycogen metabolism of mouse morulae–early blastocysts *in vivo J. Reprod. Fert.* **72**, 67-73.

Edirisinghe, W. R., and Wales, R. G. (1985). Influence of environmental factors on the metabolism of glucose by preimplantation mouse embryos *in vitro*. *Aust. J. Biol. Sci.* **38**, 411-20.

Edirisinghe, W. R., Wales, R. G., and Pike, I. L. (1984). Degradation of biochemical pools labelled with [^{14}C]glucose during culture of 8-cell and morula-early blastocyst-stage mouse embryos *in vitro* and *in vivo*. *J. Reprod. Fert.* **72**, 59-65.

Hoversland, R. C., and Weitlauf, H. M. (1981). The volume of uterine fluid in 'implanting' and 'delayed implanting' mice. *J. Reprod. Fert.* **62**, 105-9.

Khurana, N. K., and Wales, R. G. (1987*a*). Effects of oestradiol and progesterone on the metabolism of [U-^{14}C]glucose by mouse morulae and early blastocysts *in vitro*. *J. Reprod. Fert.* **79**, 267-73.

Khurana, N. K., and Wales, R. G. (1987*b*). Effects of prostaglandin E-2 and F-2α on the metabolism of [U-^{14}C]glucose by mouse embryos *in vitro*. *J. Reprod. Fert.* **79**, 275-80.

Kohler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495-7.

Kuzan, F. B., and Wright, R. W. Jr. (1982). Observations on the development of bovine morulae on various cellular and non-cellular substrata. *J. Anim. Sci.* **54**, 811-16.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cell. *Proc. Natl Acad. Sci. U.S.A.* **79**, 7634-8.

Maule-Walker, F. M., and Poyser, N. L. (1974). Production of prostaglandins by the early pregnant guinea-pig uterus *in vitro*. *J. Endocr.* **61**, 265-71.

Mitchell, J. A., and Yochim, J. M. (1968). Intrauterine oxygen tension during the estrous cycle in the rat: its relation to uterine respiration and vascular activity. *Endocrinology* **83**, 701-5.

Ozias, C. B., and Stern, S. (1973). Glycogen levels of preimplantation mouse embryos developing *in vitro*. *Biol. Reprod.* **8**, 467-72.

Paul, J. (1975). 'Cell and Tissue Culture'. 5th edn. pp. 354-84. (Churchill: London.)

Pike, I. L., and Wales, R. G. (1982). The uptake and incorporation of glucose especially into glycogen pools of preimplantation mouse embryos during *in vitro* culture. *Aust. J. Biol. Sci.* **35**, 195-206.

Potter, S. W., Buckley, P. M., and Morris, J. E. (1981). Morphological responses of mouse uterine epithelium to hormones in suspension culture. *J. Cell. Biol.* **91**, 171a.

Riehl, R. M., Pathak, R. K., and Harper, M. H. K. (1983). A reliable method for isolating endometrial epithelial cells from rabbits and preliminary studies of prostaglandin uptake. *Biol. Reprod.* **28**, 363-75.

Salamonsen, L. A., Osum, W., Doughton, B., and Findlay, J. K. (1985). The effects of estrogen and progesterone *in vivo* on protein synthesis and secretion by cultured epithelial cells from sheep endometrium. *Endocrinology* **117**, 2148-59.

Salamonsen, L. A., Doughton, B. W., and Findlay, J. K. (1986). The effects of the preimplantation blastocyst *in vivo* and *in vitro* on protein synthesis and secretion by cultured epithelial cells from sheep endometrium. *Endocrinology* **119**, 622-8.

Sherman, M. I. (1978). Implantation of mouse blastocysts *in vitro*. In 'Methods in Mammalian Reproduction'. (Ed. J. C. Daniel Jr.) pp. 81-125. (Academic Press: New York.)

Surani, M. A. H. (1977). Cellular and molecular approaches to blastocyst uterine interactions at implantation. In 'Development in Mammals'. (Ed. M. H. Johnson.) Vol. 1, pp. 245-305. (Elsevier/North Holland Biomedical Press: Amsterdam.)

Tantayaporn, P., Mallikarjuneswara, V. R., De-Carlo, S. J., and Clemetson, C. A. B. (1974). The effects of estrogen and progesterone on the volume and electrolyte content of the uterine luminal fluid of the rat. *Endocrinology* **95**, 1034-45.

Uehara, S., Villee, C. A., and Hoshiai, H. (1984). Effect of oestradiol, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ on incorporation of [^{3}H]uridine by preimplantation mouse embryos *in vitro*. *Tohoku J. Exp. Med.* **144**, 305-13.

Wiley, L. M., Yamani, S., and Muyden, D. V. (1986). Effect of potassium concentration, type of protein supplement, and embryo density on mouse preimplantation development *in vitro*. *Fert. Steril.* **45**, 111-19.

Manuscript received 15 April 1987, accepted 1 July 1987