Upsala J Med Sci 83: 135–139, 1978

Effect of the Endocrine State of Blastocyst Donors on the Time Required for Initiation of Trophoblast Outgrowth

GRELS NÆSLUND and ÖRJAN LUNDKVIST

From the Reproduction Research Unit, Biomedical Centre, Uppsala, Sweden

ABSTRACT

Blastocysts from mice in a state of delayed implantation after ovariectomy were recovered on day 5, 7 or 9 (day 1 was the day a vaginal plug was found). Blastocysts were also recovered on day 7 from animals that had received an injection of oestradiol-17β 8, 16 or 24 hours earlier. The blastocysts were incubated in a modified Brinster's medium to which serum had been added and the time of initiation of blastocyst outgrowth was recorded. Blastcysts from early delay grew out before those from late delay. A steady state of outgrowth time was achieved on day 7. It is therefore suggested that blastocysts for culture experiments in which the metabolic activity level is crucial should not be recovered before the steady state is attained. Oestrogen injections caused earlier outgrowths, at least after 16 h, which indicates that the earlier outgrowth soon after ovariectomy might also be caused by a residual effect of the oestrogen.

INTRODUCTION

Blastocyst implantation in rats and mice is determined by the endocrine state of the animal. When pregnant animals are ovariectomized on any of the first three days after mating and subsequently given progesterone, implantation will be delayed (7, 10, 20). Within a few days the blastocysts will attain a state of low metabolic activity (8, 11, 19), and show a characteristic ultrastructure (1, 13). The depression of the blastocyst activity during delay probably depends both on the exogenous administration of progesterone (9) and on the declining influence of ovarian oestrogen, since if oestrogen is given, the blastocysts will become activated and start to implant (7, 20).

Mouse blastocysts *in vitro* will grow out on the bottom of the culture dish within a few days. This process has been regarded as an analogue of implantation (6) and has been found to be associated with ultrastructural (Naeslund, G. & Nilsson, O.: To be published) and metabolic (9, 15, 16, 18) changes similar to those occurring on blastocyst activation during implantation. Since the endocrine state of the host animal determines the activity of the blastocysts *in vivo*, it is also possible that the same state of the blastocyst donors determines the activity of blastocysts *in vitro*. Knowledge of this matter is important for culture experiments, where the level of blastocyst activity is crucial.

The aim of this study was therefore to test whether the interval between the start of the culture and the beginning of outgrowth of the blastocysts is related to the endocrine state of the blastocyst donors. Mouse blastocysts were recovered at different times both during experimental delay of implantation and during oestrogen-induced activation for implantation.

MATERIAL AND METHODS

Virgin, albino mice of the NMRI strain (Anticimex, Stockholm, Sweden) were caged with males over night and the day when a vaginal plug was found was called day 1 of pregnancy. To induce experimental delay of implantation bilateral ovariectomy was performed on day 3 (20) and from that day on 1 mg of progesterone (Makor Chemicals, Jerusalem, Israel) dissolved in peanut oil (25 mg/ml) was given subcutaneously daily, the day of flushing excluded (1).

Blastocysts were recovered on day 5, 7 or 9 during delay of implantation and on day 7–8, 16 or 24 h after a s. c. injection of 0.1 μg oestradiol-17β (AB Leo, Helsingborg, Sweden) dissolved in propylene glycol (1 μg/ml). The uteri were flushed with culture medium, that had previously been equilibrated with 5% CO_2 in air overnight. During the flushing procedure the medium was collected in Petri dishes containing liquid paraffin in order to maintain the pH during the subsequent search for blastocysts. Brinster's medium for ovum culture was used (2), in which the lactate had been replaced with 1.0 mg/ml of glucose, which is appropriate from the 8-cell stage on in the mouse (3). Fetal calf serum (Flow, Ayrshire, Scotland) in a concentration of 1% was added to permit outgrowths (5).

Immediately after flushing the blastocysts were trans-

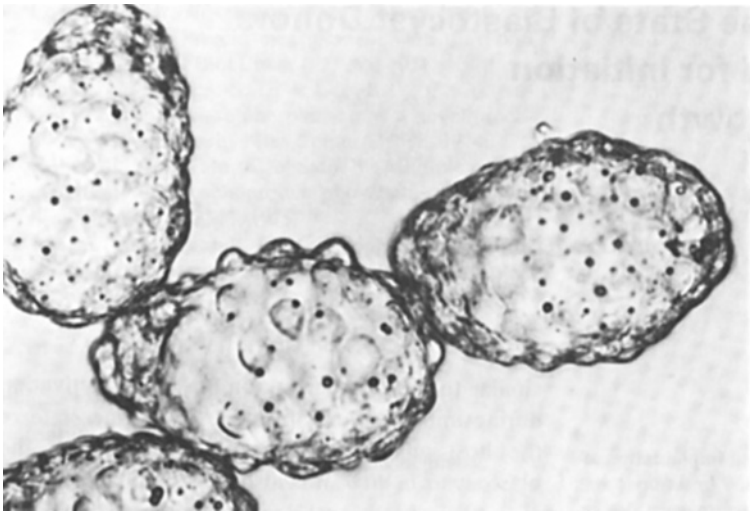

Fig. 1. Blastocysts in culture droplets 24 h after explantation; day-7 group. The blastocysts are expanded without signs of trophoblast outgrowth. Bright field. ×250.

ferred to 0.1-ml droplets of the medium under liquid paraffin by means of glass capillaries connected to an Agla micrometer syringe (Burroughs Wellcome & Co, London, England). The droplets were made the day before the culture started and were kept in plastic Petri dishes (Falcon, Oxnard, California, USA) at 37°C in an atmosphere of 5% CO_2 in air. Four to five blastocysts were placed in each droplet.

Observations of the blastocysts were made with an inverted microscope (Biovert, Reichert, Austria), bright-ground microscopy being used for screening purposes and phase contrast (magnification ×160) to detect the first signs of outgrowth (Fig. 1 and 2). The initiation of the outgrowth was defined as the moment when growing cells were first seen outside the blastocyst contour. The observations were made every 8 h and always by the same person. The first observation of an outgrowth was recorded as occurring in the middle of the previous 8-hour interval. Analysis of variance was used to detect significant differences between the experimental groups.

RESULTS

The frequency distributions for the moment of initiation of blastocyst outgrowth are summarized in Fig. 3. The means for the groups, and the number of animals, blastocysts, and droplets are given in Table I. The means for every droplet were used as observations in the subsequent tests of significance (Table II).

The duration of the delay influenced the moment of initiation of outgrowth, as the blastocysts from day 5 started to grow out 13 h earlier, on the average, than the blastocysts from day 7, a statistically significant difference, whereas the difference between day-7 blastocysts and those from day 9 was non-significant. Of the blastocysts from day 5, those encased in zona pellucida grew out earlier

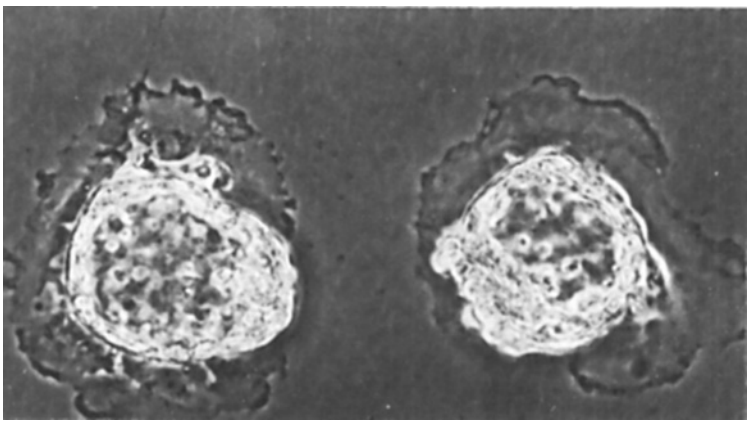

Fig. 2. Outgrowing trophoblast cells around two blastocysts 64 h after explantation; day-7 group. Phase contrast. ×250.

Table I. *Time required for initiation of outgrowth in vitro of blastocysts from donors in various endocrine states*

	Group	Hours after initiation of culture ($M \pm$S.D.)	No. of animals	No. of blasto-cysts	No. of droplets
Delay of implantation	Day 5	44± 9	19	33	8
	Day 7	57±10	40	97	21
	Day 9	61± 7	40	118	24
Oestrogen activation	Oestrogen 8 h	60± 4	6	32	6
	Oestrogen 16 h	47± 8	14	22	5
	Oestrogen 24 h	9± 4	8	29	6

(mean 39 h) than the zona-free ones (mean 47 h). No tests of significance were performed, because of the small number of zona-encased blastocysts.

Oestrogen injected into the donor mice had a marked effect on the blastocysts *in vitro* compared with those from day 7 of delay. No difference in the appearance of outgrowths was seen 8 h after the injection, whereas after 16 h there was a statistically significant difference. The effect was considerable 24 h after the injection, as the blastocysts grew out on the average 48 h earlier than the day-7 blastocysts from animals in a state of delay.

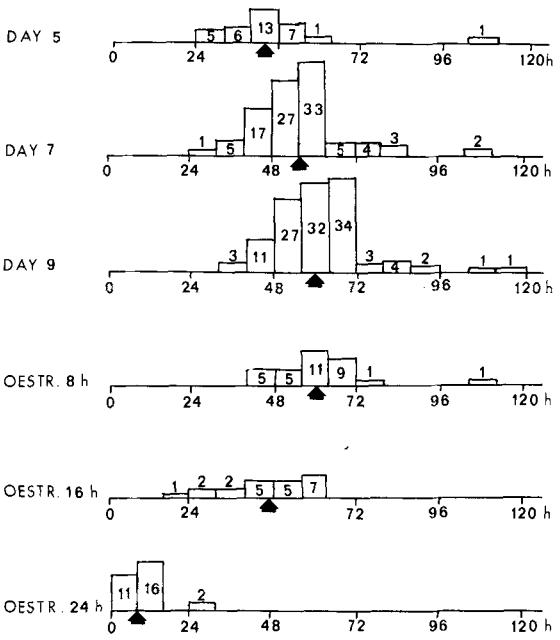

Fig. 3. Histograms representing the frequency distributions for the appearance of the first outgrowth. The figures within or above rectangles represent the number of blastocysts that started to grow out during each 8-hour interval. Arrows indicate means. Day 5: Blastocysts recovered on day 5 during delay of implantation. Day 7: Blastocysts recovered on day 7 during delay of implantation. Day 9: Blastocysts recovered on day 9 during delay of implantation. Oestrogen 8 h: Blastocysts recovered on day 7, 8 h after an oestrogen injection. Oestrogen 16 h: Blastocysts recovered on day 7, 16 h after an ostrogen injection. Oestrogen 24 h: Blastocysts recovered on day 7, 24 h after an oestrogen injection.

DISCUSSION

Blastocyst activity has often been determined by measuring specific metabolic steps, such as the incorporation of RNA precursors (4, 12, 18) and of amino acids (16, 19) and the production of carbon dioxide (9, 14, 15). In the present experiment a new method was tried—determination of the time required for initiation of trophoblastic outgrowth *in vitro*. This method supplements others by taking into account the proliferating capacity of trophoblast cells. The technique is simple and easy to handle, which is an advantage when several experimental groups are to be tested.

The moment of initiation of blastocyst outgrowth was estimated by microscopic observations of the blastocyst cultures. However, the very first cells

Table II. *Significance levels for the differences between groups*

Group	Significance level
Day 5 vs. day 7	$P<0.005$
Day 7 vs. day 9	Non-significant
Day 7 vs. oestrogen 8 h	No apparent difference
Day 7 vs. oestrogen 16 h	$P<0.05$
Day 7 vs. oestrogen 24 h	A manifest difference

growing out are difficult to distinguish, but as trophoblast cells proliferate rapidly, judging from the changes that occurred in the same outgrowth area between two observations, the influence of this error is probably of minor importance. The sensitivity of the method depends upon the length of the interval between two observations. Too close observations might disturb the growth of the blastocysts and in the present experiment an interval of 8 h was found suitable, which resulted in an accuracy of ±4 h for every single observation.

The reliability of this method was judged to be adequate for the present purpose, as no statistically significant differences were observed between various batches of day-7 blastocysts cultured during different periods of the experiment. It is therefore concluded that the present technique of measuring the time required for blastocysts to start their outgrowth *in vitro* can be useful for evaluating factors that influence the blastocyst activity.

The results demonstrate that the activity of the blastocyst *in vitro* is, in fact, influenced by the endocrine state of the blastocyst donor. Thus, delayed blastocysts flushed soon after the ovariectomy grew out faster than those flushed a few days later. However, from day 7 onwards (i.e. after 4 days diapause) no difference were observed between the groups of delayed blastocysts (Table I). The conclusion may therefore be drawn that the slow attainment of a steady state of blastocyst ultrastructure (1, 13) and metabolic activity (11) during delay corresponds to a similar attainment of activity *in vitro*.

When oestrogen was injected into the donor animals, their blastocysts grew out faster *in vitro*. This effect was more marked the longer after the oestrogen injection the blastocysts were recovered. Further, blastocysts from day 5 of normal, non-delayed pregnancy will grow out faster than those from animals during delay of implantation (Naeslund, G.: Unpublished observation). Thus, if blastocysts are influenced by oestrogen before being cultured the time required for initiation of outgrowth *in vitro* will change. Therefore, in culture experiments where the level of blastocyst activity is crucial, it is important to define strictly the endocrine state of the blastocyst donors and the time of recovery. Furthermore, if a basic level is desired, the present experiment suggests that delayed blastocysts should not be recovered earlier than day 7.

The outgrowth of trophoblast cells *in vitro* has been regarded as an analogue of the attachment and invasion of trophoblast cells *in vivo* (6). However, the blastocyst *in vitro* lives in a totally different environment and faces quite a different material for growth than the blastocyst in the uterine cavity. One possible way of determining whether the cells of the blastocyst respond differently to these two conditions is to compare the intracellular changes *in vitro* with those *in vivo*. This kind of study requires well defined culture conditions to avoid irrelevant influences on the morphology. The present design for blastocyst culture seems suitable for this purpose and will be used in future experiments for ultrastructural comparisons of blastocyst developments *in vivo* and *in vitro* (Naeslund, G. & Nilsson, O.: To be published) and for testing some hypotheses on the control of blastocyst growth.

ACKNOWLEDGEMENTS

We thank Mrs Barbro Einarsson for skilful technical assistance and Dr Gunnar Ekbohm for statistic consultation. The study was financially supported by the Swedish Medical Research Council (Project No. 12X–70 to Professor Ove Nilsson) and the Swedish Foundation for Prenatal Research.

REFERENCES

1. Bergström, S.: Delay of blastocyst implantation in the mouse by ovariectomy or lactation. A scanning electron microscope study. Fertil Steril *23*: 548, 1972.
2. Brinster, R. L.: A method for *in vitro* cultivation of mouse ova from two-cell to blastocyst. Exp Cell Res *32*: 205, 1963.
3. Brinster, R. L. & Thomson, J. L.: Development of eight-cell mouse embryos *in vitro*. Exp Cell Res *42*: 308, 1966.
4. Gulyas, B. J. & Daniel, J. C., Jr: Incorporation of labeled nucleic acid and protein precursors by diapausing and nondiapausing blastocysts. Biol Reprod *1*: 11, 1969.
5. Gwatkin, R. B. L.: Defined media and development of mammalian eggs *in vitro*. Ann NY Acad Sci *139*: 79, 1966.
6. — Amino acid requirements for attachment and outgrowth of the mouse blastocyst *in vitro*. J Cell Physiol *68*: 335, 1966.
7. Humphrey, K. W.: The induction of implantation in the mouse after ovariectomy. Steroids *19*: 591, 1967.
8. MacLaren, A.: Blastocyst activation. *In* The Regulation of Mammalian Reproduction (ed. S. J. Segal, R. Crozier, P. A. Corfman & P. G. Condliffe), pp. 321–328. Charles C. Thomas, Springfield, Ill., 1973.
9. MacLaren, A. & Menke, T. M.: CO_2 output of mouse blastocysts *in vitro*, in normal pregnancy and in delay. J Reprod Fertil (Suppl.) *14*: 23, 1971.

10. Mayer, G.: Delayed nidation in rats: A method of exploring the mechanisms of ovo-implantation. *In* Delayed Implantation (ed. A. C. Enders), pp. 213–231. Univ. Chicago Press, Chicago, 1963.
11. Menke, T. M.: Changes in mouse blastocyst carbon dioxide production as a function of time postcoitum in delay of implantation during lactation or following ovariectomy. Biol Reprod *7*: 414, 1972.
12. Mohla, S. & Prasad, M. R. N.: Early action of oestrogen on the incorporation of [^{3}H]uridine in the blastocyst and uterus of rat during delayed implantation. J Endocrinol *49*: 87, 1971.
13. Nilsson, O.: The morphology of blastocyst implantation. J Reprod Fertil *39*: 187, 1974.
14. Torbit, C. A. & Weitlauf, H.M.: The effect of oestrogen and progesterone on CO_2 production by 'delayed implanting' mouse embryos. J Reprod Fertil *39*: 379, 1974.
15. — Production of carbon dioxide *in vitro* by blastocysts from intact and ovariectomized mice. J Reprod Fertil *42*: 45, 1975.
16. Weitlauf, H.M.: *In vitro* uptake and incorporation of amico acids by blastocysts from intact and ovariectomized mice. J Exp Zool *183:* 303, 1973.
17. — Metabolic changes in the blastocysts of mice and rats during delayed implantation. J Reprod Fertil *39*: 213, 1974.
18. — Effect of uterine flushings on RNA synthesis by 'implanting' and 'delayed implanting' mouse blastocysts *in vitro*. Biol Reprod *14*: 566, 1976.
19. Weitlauf, H. M. & Greenwald, G. S.: A comparison of ^{35}S methionine incorporation by the blastocysts of normal and delayed implanting mice. J Reprod Fertil *10*: 203, 1965.
20. Yoshinaga, K. & Adams, C. E.: Delayed implantation in the spayed, progesterone treated adult mouse. J Reprod Fertil *12*: 593, 1966.

Received January 18, 1978

Address for reprints:

G. Næslund
Department of Anatomy
Biomedical Centre
Box 571
S-751 23 Uppsala
Sweden