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The Effect of Glucose-, Arginine- and Leucine-deprivation on Mouse Blastocyst Outgrowth *In Vitro*[1]

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ABSTRACT

To estimate the degree of trophoblast outgrowth in vitro, mouse blastocysts obtained after delay of implantation were cultured either in a modified Brinster medium or in the same medium with exclusion of various combinations of glucose, arginine and leucine. Trophoblast outgrowth was prevented only in a medium from which all three substances were excluded. In this medium the blastocysts remained expanded for 5 days without signs of trophoblast outgrowth - a growth arrest in vitro.

After transfer of blastocysts growth arrested in vitro for 5 days to a complete medium including both glucose and the two amino acids, normal outgrowths occurred within two or three days. The growth-arrested blastocysts also developed normally for at least one week when transplanted into salpingectomized foster mothers. It is concluded that blastocyst activation in vitro can be controlled by a few nutrients in a way reminiscent of the activation prior to implantation in utero.

Blastocysts activated in utero by systemic administration of oestrogen for various lengths of time before the start of culture, or in vitro by preincubation in a medium containing glucose and all amino acids, also grew out in the growth arrest medium if they had been activated for a sufficiently long time, 18 h in utero and 1 h in vitro, thus indicating that when a blastocyst has reached a certain degree of activation its growth can not be arrested by exclusion of glucose, arginine and leucine.

INTRODUCTION

Activation of a uterine mouse blastocyst by systemic administration of oestrogen during experimental delay of implantation might be due, for instance, either to disappearance of a uterine inhibitor (20), or to an increase of

[1]Part of this study was presented at the Society for the Study of Fertility, Sheffield, England, 1976.

nutrients in the uterine secretion (9,13). Similar ideas can be applied to conditions in vitro. Thus, when blastocysts in delay are transferred from the uterine cavity to a medium containing serum, glucose and amino acids, the trophoblast cells become activated (15) and grow out (8), indicating either that the blastocyst gets released from the uterine inhibitor or that on transfer to the culture medium it is supplied with nutrients that are lacking in the uterus (25).

The uterine secretion, which is assumed to harbour the factors controlling mouse blastocyst activity during implantation, is increased a few hours after an oestrogen injection (17). Further, its composition is changed around the time of implantation according to various assays of proteins and carbohydrates.

The total protein content in rat and mouse uterine secretion has thus been found to increase and the presence of new serum and non-serum proteins has been observed (2,24). It is evident that in vitro, as in utero, serum or a few other macromolecular components are necessary for outgrowth (7).

Fructose is present in increasing amounts when the roe deer blastocyst in diapause starts implanting (1), whereas in the mouse, studies suggest that glucose is the more important carbohydrate for implantation (19). In vitro, glucose has also been found necessary for hatching of zona-encased mouse blastocysts from superovulated mice (28). However, experiments on mice during delay of implantation have shown that even when glucose is excluded from the culture medium blastocyst outgrowths are obtained (15).

In vitro, amino acids have also been found important, as they affect blastocyst hatching, attachment and outgrowth and thus may be of significance for implantation. In one study blastocysts could be kept expanded without trophoblast outgrowth when arginine or leucine was excluded from the medium (8), but with another experimental set-up these amino acids only inhibited outgrowth to some extent (23). In utero the concentrations of various amino acids, including arginine and leucine, were the same at implantation and during delay (9).

Since there are indications that glucose, arginine and leucine are critical nutrients, the present experiments were undertaken to test the influence of exclusion of glucose and the two amino acids from the culture medium on the outgrowth of delayed mouse blastocysts, in order to find out whether growth arrest reminiscent of delayed implantation could also be obtained in vitro. Since this purpose was achieved, blastocysts in various degree of activation were also evaluated to examine whether a critical stage of blastocyst activation exists beyond which the blastocyst loses its capability of being growth-arrested in vitro.

MATERIAL AND METHODS

Brinster's medium for ovum culture (3) with replacement of lactate by 1.0 mg/ml of glucose, which is regarded appropriate from the 8-cell stage onwards (4), was used as control medium. The amino acids of Eagle's Basal Medium (5) stored in two stock solutions with 100 times the final concentration, one with cystine and tyrosine dissolved in 0.1 N HCl and one with the other amino acids, dissolved in water. Glutamine was added as dry powder. Foetal calf serum (Flow Laboratories, Irvine, Scotland), dialysed against 100 times its volume of isotonic NaCl at $4^{\circ}C$ with two changes daily for three days to remove glucose and amino acids, was used within a week after the dialysis (8). Reagent grade water from a milli-Q2 System (Millipore Corp., Bedford, Mass., U.S.A.) was used. This water had a specific resistance of about 18 MΩ/cm.

Six types of media, all differing with regard to the presence of the three components glucose, arginine and leucine (Fig. 1), were employed. These were: 1. A control medium containing glucose and all amino acids. 2. An experimental medium without addition of glucose. 3. An experimental medium without addition of arginine and leucine. 4. An experimental medium without addition of glucose and arginine. 5. An experimental medium without addition of glucose and leucine. 6. An experimental medium without addition of glucose, arginine or leucine. All media contained 1 % dialysed foetal calf serum, except in experiments where the effect of various serum concentrations were tested.

Virgin, albino mice of the NMRI strain (Anticimex, Stockholm, Sweden) were caged with males overnight and the day on which a vaginal plug was found was called day 1 of pregnancy. Ovariectomy was performed on day 3 to induce delay of implantation and 1 mg of the depot preparation medroxiprogesterone (Depo-Provera, Upjohn Co, U.S.A.) was given subcutaneously at ovariectomy and again 5 days later (30). The normal animals were taken for experimentation on the afternoon of day 4, while the animals in delay of implantation were used on day 8-12, as a steady state of outgrowth activity is attained on day 7 (16). The uterine horns were flushed with phosphate-buffered saline (PBS) containing magnesium and calcium (SBL, Stockholm, Sweden) with addition of 1% dialysed foetal calf serum to prevent the blastocysts from becoming adhesive. The culture medium was equlibrated against an atmosphere of 5% CO_2 in air for at least one hour and droplets of the medium with a volume of 0.1 ml were then covered with liquid paraffin (3) and kept in plastic tissue culture dishes (Type 3001F, Falcon, Oxnard, U.S.A.) at $37^{\circ}C$ in an atmosphere of 5% CO_2 in air.

As a low oxygen tension has been observed in the rat uterus around the time of implantation, and as changes in oxygen tension might influence carbohydrate metabolism (29), tests on the effect of a low-oxygen milieu were

also carried out by using airtight jars containing the culture dishes and flushed with a mixture of 5% CO_2 and 5% O_2 in N_2 and then closed with a slight residual gas pressure. The jars were placed in a culture box at a temperature of 37°C and the dishes were checked daily. If some pressure was still left the gas atmosphere was considered unchanged.

The blastocysts were transferred to the droplets, about 5 in each, by means of glass capillaries connected to an Agla micrometer syringe (Burroughs Wellcome and Co., London) and were then observed daily for 5 days in an inverted microscope (Biovert, Reichert, Austria). Phase-contrast microscopy (magnification x 160) was used to detect the first signs of outgrowth (16).

To examine the influence of various media on the outgrowth, blastocysts from mice in delay of implantation were cultured in media from which glucose and/or amino acids had been excluded. In order to check the capacity for subsequent outgrowth in blastocysts whose growth had been arrested in vitro, the medium without glucose, arginine or leucine was exchanged for the control medium.

To check their ability to implant, blastocysts from delay of implantation that had been growth-arrested in vitro for 6 days were transferred to the control medium for 24 h to activate them, and were then transplanted in PBS and 5 % dialysed serum to the left uterine horns of NMRI mice on day 4 of pregnancy. The mice had been bilaterally salpingectomized on day 2. Uterine swellings observed after a week were embedded in paraffine and sectioned for light microscopy.

To examine whether a critical stage of blastocyst activation exists beyond which the blastocyst loses its ability to be growth-arrested in vitro, experiments were performed in which blastocysts from delay of implantation were activated either in vitro or in utero before culture in the growth-arrest medium. For activation in vitro, blastocysts were pre-incubated for 1, 3 or 6 h in the control medium, whereas for activation in utero blastocysts were taken from animals that had received 0.1 µg of 17-β-oestradiol 12, 18 or 24 h before the start of culture (Fig. 3).

RESULTS

The blastocysts from animals in delay of implantation generally became contracted during the process of recovery and transfer to the droplets, probably because they were zona-free, but in the control medium they expanded within 24 h and within three days the trophoblast cells of the blastocysts had grown out producing a pile of cells (Fig. 1).

In media without glucose and/or amino acids the outgrowth was affected. With exclusion of glucose alone the blastocysts became expanded within 24 h

but the first outgrowths did not appear until days 3 to 5. After 5 days all blastocysts had grown out, the outgrowths being smaller, however, than in the control medium. With exclusion of arginine and leucine, exclusion of glucose and arginine and exclusion of glucose and leucine, some of the blastocysts grew out on day 4 to 5, while the others remained expanded, but showed no trophoblast outgrowth (Fig. 1). When all three nutrients - glucose, arginine and leucine - were excluded from the medium, all blastocysts still remained expanded without any signs of trophoblast outgrowth after 5 days in culture - a growth arrest in vitro.

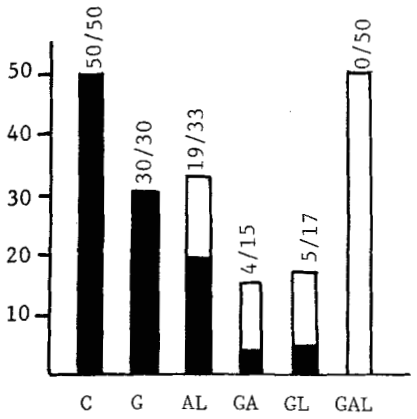

Fig. 1. No. of outgrowing blastocysts to total number of blastocysts after 5 days in culture. Two to four replicate experiments were performed in each group. The height of the filled bars indicates the number of blastocysts, that had grown out after 5 days' culture. The remaining blastocysts were expanded. "C" indicates the control medium; the other bars represent experimental media with exclusion of different components. (G = glucose, A L = arginine and leucine G A = glucose and arginine, G L = glucose and leucine, G A L = glucose, arginine and leucine).

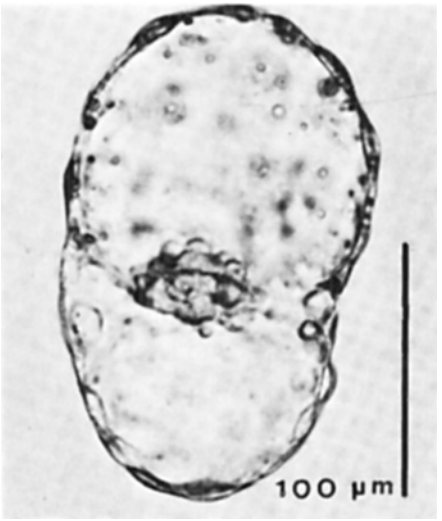

Fig. 2. A blastocyst growth-arrested for 5 days in a medium from which glucose, arginine and leucine were excluded. The blastocyst is expanded and somewhat rugged in outline. The embryoblast has partly loosened from the inner surface of the trophoblast. Bright field microscopy.

Most of the blastocysts in all types of media - the control medium as well as the media from which nutrients were excluded - had become attached to the plastic surface within a day and remained so throughout the time of culture. In a medium containing neither glucose, arginine nor leucine the longest diameter of the blastocysts slowly increased from about 100 µm after 24 h to about 150-200 µm after 5 days and the blastocyst contour became somewhat rugged (Fig. 2).

The embryoblast of most blastocysts showed a peculiar change after 2 to 3 days, in the form of loosening of most of the contact with the inner surface of the trophoblast, leaving only attachment with thin connections from the embryoblast edges (Fig. 2). This change was observed in all five types of experimental media with exclusion of nutrients.

The viability of the trophoblasts of the blastocysts growth-arrested in vitro in the medium with glucose, arginine and leucine excluded was tested by changing the experimental medium for the control medium after 5 days. It was then observed that outgrowths with a normal appearance and size occurred 2 to 3 days after the change of medium (30 blastocysts from three replicate experiments).

The viability of the embryoblasts was tested by transplantation experiments. Out of 23 blastocysts growth arrested in vitro for 6 days in the medium with glucose, arginine and leucine excluded and transplanted to the left uterine horns of ten bilaterally salpingectomized recipients in two replicate experiments, 5 animals had uterine swellings after one week. Three of these showed only decidual tissue, while the other 2 contained embryos with a normal appearance (neural tube and primitive heart).

Blastocysts from day 4 of normal pregnancies, in one experiment, sometimes hatched (2/10 blastocysts), and zona-free blastocysts always grew out when glucose alone was excluded and 1% dialysed serum was added. Also with 10% dialysed serum, as in Wordinger's and Brinster's study (28), 4/10 blastocysts hatched and all those that hatched grew out on day 3 to 5. In a medium from which glucose, arginine and leucine were all excluded, however, all 15 blastocysts had hatched after 3 to 4 days and subsequently remained expanded and relatively large without any signs of trophoblast outgrowth (two replicate experiments).

A few variants of the culture system were tried. An atmosphere containing only 5 % O_2 with 5 % CO_2 in N_2 instead of the usual 20 % O_2 (air) did not change the effect of the various media on the outgrowth (5 to 8 blastocysts in each group, two replicate experiments). The expanded blastocysts cultured in a medium deprived of glucose, arginine and leucine in a low oxygen milieu appeared similar to those cultured in 20% oxygen. When serum was excluded from the control medium the blastocysts contracted after 2 to 3 days (10

blastocysts from two replicate experiments), whereas exclusion of serum from the medium containing no glucose, arginine of leucine did not cause contraction; the blastocysts were still expanded for one to 2 weeks and the embryoblast loosened just as in the presence of serum (20 blastocysts from two replicate experiments). When the concentration of dialysed serum was increased to 20 %, 3 of 10 blastocysts grew out even when all three nutrients were excluded (two replicate experiments).

Experiments with blastocysts at various degrees of activation showed that blastocysts that had been preincubated in the control medium for 1 h or longer or taken from animals that had received oestradiol 18 h earlier were able to grow out in the medium without glucose, arginine or leucine (Figs. 3 and 4). The outgrowths after 1 and 3 h preincubation in the control medium were small and did not appear until after 5 days in the growth-arrest medium.

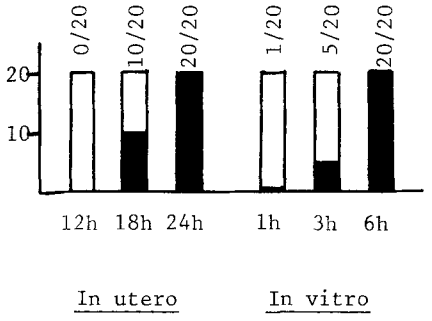

Fig. 3. Capability of the growth arrest medium (with glucose and arginine-leucine excluded) to impede outgrowth of blastocysts activated in utero or in vitro. They were activated in utero by giving 0.1 µg 17-β-oestradiol to the animal 12, 18 or 24 h before start of culture and in vitro by incubating them in the control medium 1, 3 or 6 h before change of culture medium. Two replicate experiments in each group. Concerning filled and unfilled bars see Fig. 1.

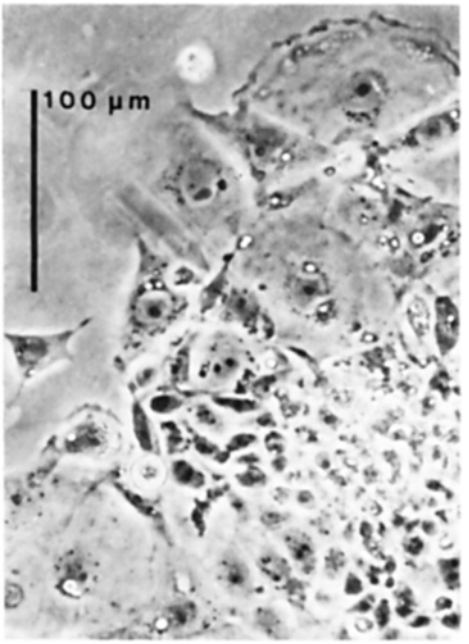

Fig. 4. Trophoblast outgrowth from a blastocyst activated by 0.1 µg 17-β-oestradiol 24 h before start of culture and then incubated for 5 days in a medium with glucose, arginine and leucine excluded. Phase contrast microscopy.

DISCUSSION

When using trophoblast outgrowth as a measure of the growth-promoting potential of various culture media, it is an advantage to use blastocysts from animals in delay of implantation, as they are zona-free (12) and have a defined capacity for outgrowth (16). In the present experiments the blastocysts were observed for 5 days, because in a medium permitting normal outgrowth most delayed blastocysts grow out within 3 days and only few later (16). Since in earlier assays of the trophoblast outgrowth no such standardized systems have been used, comparisons of the various results should be made with caution.

The proliferation of animal cells in culture - quite generally - can be stimulated by a variety of substances. For some such substances, like serum and specific growth-stimulating factors, the rate of proliferation is a function of the concentration (26,27), and also in the case of others, like amino acids, proliferation is now thought to depend on the concentration (11). The present experiments focus on the influence of this type of substance on blastocyst growth and viability.

The exclusion of arginine and leucine from a blastocyst culture medium has been reported by some authors to result in a total inhibition of trophoblast outgrowth (8). Others, however, have found less complete inhibition when the two amino acids are omitted (23). These discrepancies might be due to the use of different materials for cell support (glass versus collagen) or to the culture of different types of blastocysts (cultured from 2-cell stage versus flushed on day 4). However, since it was also found in the present experiments with blastocysts delayed in utero that exclusion of arginine and leucine only inhibited outgrowth to some extent, these amino acids are probably not the only growth controlling factors.

Glucose exclusion from a medium has been reported to almost totally inhibit hatching of zona-encased blastocysts (28). The degree of outgrowth, however, might have been difficult to test in the model used, as the blastocysts, which derived from superovulated animals, rarely hatched and thus very few had the opportunity to grow out. In these experiments the blastocysts were further cultured on collagen and the observation time was only 48 h. Consequently the small, late appearing outgrowths that were observed in the present study might have been missed with this experimental set-up. In the present experiments, the growth of normal day-4 blastocysts was tested in a medium from which only glucose was excluded, those that hatched always grew out. Neither were blastocysts obtained during diapause completely inhibited in media without glucose, and therefore lack of glucose in the uterine secretion is not likely alone to cause a delay in utero.

Media from which all three nutrients - glucose, arginine and leucine - were excluded allowed the blastocysts to remain expanded, i.e. their growth was arrested, reminiscent of blastocysts delayed *in utero*. The blastocysts were observed regularly for 5 days, but some blastocysts kept *in vitro* for a longer time remained expanded for about two weeks, though they became more irregular in outline. When the excluded components were added, the blastocysts were activated and outgrowths occurred after two to three days corresponding to the response by blastocysts in uterine days corresponding to the response by blastocysts in uterine diapause when transferred to a complete medium (16). Since the shape and size of the outgrowths were similar in both cases, the trophoblast cells were probably undamaged by the exclusion of glucose and the two amino acids. Ultrastructural studies are in progress with the aim of shedding further light on growth-arrest of blastocysts *in vitro*.

Blastocysts whose growth was arrested *in vitro* increased their diameter slowly. This expansion could explain why day-4 blastocysts, only some of which hatched in a medium deprived only of glucose, always hatched when both glucose and the two amino acids were excluded.

The observed loosening of the embryoblast could be due to the loose connection between trophoblast and embryoblast (14), in combination with the enlargement of the blastocyst in the growth-arrest medium. It might also indicate an adverse effect of the growth-arrest medium on this part of the blastocyst, since the embryoblast is more dependent on the amino acid concentration than the trophoblast (23). However, the blastocysts that were growth-arrested *in vitro* developed normally for at least one week when transplanted to salpingectomized pregnant mice. Sherman and Barlow (21) reported that blastocysts in media with exclusion of only arginine and leucine survived a transplantation for about a week. In the present experiments no attempt was made to standardize the transplantation technique which would have been necessary in order to correlate the success rate with that of blastocysts not cultured. The transplantation results indicate, however, that both embryoblast and trophoblast could survive in the growth arrest medium.

Experiments with activated blastocysts showed that the growth-arrest medium did in fact allow trophoblast outgrowth from blastocysts activated by systemic oestrogen or by previous incubation in a complete medium, where blastocysts become metabolically activated (15). This suggests that the growth-arrest is due to an absence of sufficient activation *in vitro* and not to a hostile environment making outgrowth impossible. When the blastocyst has reached a certain degree of activation its growth could no longer be arrested *in vitro*. The necessary activation time was 18 h *in utero* and only 1 h *in vitro* perhaps reflecting the time lag between oestrogen administration and

production of uterine secretion. As blastocysts are able to grow out in a growth-arrest medium provided they are sufficiently activated, use of this experimental system might be one simple way to determinate the degree of blastocyst activation.

Attachment of the blastocysts to the surface has been considered a stage indicating a subsequent outgrowth (8). Blastocysts from delay of implantation cultured in a complete medium have been observed first to attach and then to loosen from the substratum before the start of outgrowth, indicating changes in the characteristics of the blastocyst surface (22). Also with the present experimental set up this phenomenon was observed in the control medium. The type of surface probably has a profound effect on attachment. Moreover, the definition of attachment as the capacity of the blastocysts to adhere to the solid surface even also when the culture dish is moved (23) is rather unprecise. In the present experiments blastocysts attached even in a growth-arrest medium, indicating a poor correlation between attachment and outgrowth. In utero the blastocyst coat in delay has a negative electrical surface charge which, probably because of changes in the surface coat, decreases before implantation, thus facilitating attachment (18). The surface coat of a blastocyst in vitro, however, might behave differently, thus explaining why growth-arrested blastocysts are as firmly attached to the substratum as outgrowing ones. Experiments with surface indicators such as Alcian Blue and Con-A on blastocysts in vitro are under way.

The serum used in the medium represents an undefined factor. Normal blastocyst outgrowth has, however, been observed in serum-free media when the blastocysts have grown on a collagen surface, which should indicate that protein from serum is necessary only to make the plastic surface physically suitable (10). However, a special cell spreading factor for different types of cells have also been found in fetal calf serum (6) and might also be required for blastocysts in vitro (7). When, in the present experiments, the serum concentration was increased, some blastocysts grew out even in the growth-arrest medium, suggesting that components present in dialysed serum might activate blastocysts when present in a high concentration but not in a lower concentration. Alternatively, a high serum concentration modifies the plastic surface so that even a partly growth-arrested blastocyst can proliferate. The present observation that blastocysts whose growth had been arrested in vitro were able to expand without serum, whereas blastocysts in a complete medium became contracted in a serum-free milieu, indicates that serum is important mainly for the outgrowing blastocyst.

The implication from the present experiments for conditions in utero is that absence or a low concentration of both glucose and amino acids could keep a blastocyst in a state of delay until the missing nutrients are produced

by the endometrium. There might, However, be different ways in which growth of a blastocyst, is arrested. It is possible that *in utero* an arrest may occur due to absence of macromolecular components, and *in vitro* due to other factors. For example blastocysts delayed *in utero* are arrested in another phase of the cell cycle than blastocysts in media from which only arginine and leucine are excluded (21).

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