In vitro inhibition of trophoblast maturation and expansion of early rat blastocysts by an oestrogen antagonist

S. K. Roy¹, J. Sengupta², B. C. Paria³ and S. K. Manchanda⁴

Department of Physiology, All India Institute of Medical Sciences, New Delhi - 110029

Abstract. Rat blastocyst development and expansion were studied in an in vitro culture system. Early blastocysts collected from rats on the morning of day 5 were shown to develop normally and underwent expansion when placed in bovine serum albumin supplemented culture medium for 12 h at 37°C. Nafoxidine, an oestrogen antagonist, prevented embryo expansion when added to the culture medium, and it was found that if oestradiol-17β was added to this medium this inhibitory effect was removed and the embryos again expanded. Ethanol, used as a solvent for steroids, and cholesterol were added to the medium containing nafoxidine but the blastocysts still remained unexpanded after 12 h in culture. These results suggest a need for oestrogen action during blastocyst expansion in vitro. Cultured rat blastocysts were subjected to histochemical study for acid phosphatase (ACPase) and leucylnaphthylamidase (LNAse). In unexpanded blastocysts ACPase was found to be low while LNAse activity was higher when compared with enzyme distribution patterns in expanded blastocysts.

During development of pre-implantation mammalian embryos the formation of the trophoblast and inner cell mass and subsequent expansion of the blastococele are two important events in differentiation. During this stage a number of enzymological changes have been detected and tissue specific proteins have been identified in cells of ICM and trophoblast (Epstein 1975; Van Blerkom et al. 1976). It has been shown that blastocysts undergo expansion prior to implantation. Recently we have shown that rat morulae failed to develop when grown in vitro in medium containing nafoxidine (Roy et al. 1981). In the present study we have investigated the role of endogenous oestrogen in controlling in vitro maturation and expansion of rat blastocysts. Studies have also been performed to observe the activities of two lysosomal marker enzymes in blastomeres during expansion.

Material and Methods

Animals and collection of embryos

Adult, virgin Wistar strain rats (150–200 g body weight) were kept under a controlled light:dark schedule of 14:10. Four consecutive cycles were checked from vaginal smears and only animals exhibiting regular 4-day cycles were selected for the experiments. On the evening of pro-oestrus, females were paired with fertile males and checked next day morning for vaginal spermatozoa, the presence of which was considered to indicate the first day of pregnancy.

On day 5, at 06.00 h, rats were killed, uteri were removed and flushed with rat embryo culture medium. Early blastocysts with only the beginning of a blastocoele were collected in a drop of fresh culture medium and kept in a warm (37°C) and humid chamber under a gas phase of 5% CO₂, 5% O₂ and 90% N₂ until further use.

Culture condition and experimental protocol

Medium for rat embryo culture was prepared in the laboratory as described by Whitten (1971). We have shown that 8-cell rat embryos grow well into expanded blastocysts when cultured in this medium (Roy et al. 1981). Early rat blastocysts were washed four times in

¹ Post-doctoral research fellow of Family Planning Foundation (FPF).
² Research career development awardee of the FPF.
³ Junior research fellow of the Indian Council of Medical Research.
⁴ Professor-in-charge of the FFP project.
fresh culture medium and then randomly distributed to tubes containing 1 ml culture medium. The tubes were grouped as follows:

Group I: medium containing 50 µl ethanol-saline (1.4 × 10⁻² mM ethanol).
Group II: medium containing 3 µg nafoxidine.
Group III: medium containing 3 µg nafoxidine and 1 µg (3.4 × 10⁻³ mM) oestradiol-17β (Sigma) in 50 µl ethanol-saline (1.4 × 10⁻² mM ethanol).
Group IV: medium containing 3 µg nafoxidine and 1 µg cholesterol (E. Merck, 2.6 × 10⁻³ mM) in 50 µl ethanol-saline.
Group V: medium containing 3 µg nafoxidine and 50 µl ethanol-saline.

Embryos were cultured for a period of 12 h at 37°C in a Dubnoff shaking incubator.

Preparation of steroid and antisteroid solutions
A stock solution of 1 mg (3.6 mM) oestradiol-17β was prepared in absolute ethanol. The stock was diluted with sterile saline to obtain 20 µg/ml (7.3 × 10⁻³ mM) oestradiol solution containing 4.2 × 10⁻⁴ mM ethanol. Fifty µl of this solution was added to 1 ml of culture medium. The final ethanol concentration in the culture medium was 1.4 × 10⁻² mM and oestradiol-17β, 1 µg (3.4 × 10⁻³ mM). This concentration of ethanol was found to have no deleterious effect on rat embryo development in vitro. Saline vehicle added to the control tubes also contained a similar concentration of ethanol. An alcoholic stock solution was made with 1 mg cholesterol (2.6 mM) per ml ethanol and was diluted with sterile saline to obtain 20 µg per ml (5.1 × 10⁻² mM) cholesterol containing 4.2 × 10⁻² mM ethanol. Fifty µl of this solution was added to 1 ml of medium similar to that of oestradiol-17β. The final concentration of cholesterol in the medium was 2.6 × 10⁻⁴ mM. The non-steroidal anti-oestrogen, nafoxidine-HCl (U11, 100A, Upjohn Co. USA; 1-2 (p-(3,4-dihydro-methoxy-2-phenyl-1-naphthyl) phenoxyethyl) pyrroliodone hydrochloride) was dissolved in a small volume of acetone and diluted with sterile saline to prepare a 1 mg/ml solution of the anti-oestrogen. Acetone was later removed completely through evaporation.

**Histochemical analysis of acid phosphatase (ACPase) and leucynaphthylamidase (LNAse)**
At the termination of culture, blastocysts were recovered in glass cavity slides and the degree of expansion was first checked under a Bausch and Lomb stereomicroscope and then photographed in a Leitz research microscope. Blastocysts were then used for various other investigations including the histochemical study of ACPase (E.C.3.1.3.2) and LNAse (E.C.3.5.1.1) which are reported here. Embryos kept for ACPase were washed in 0.1 m ice-cold acetate buffer, pH 5, and stained according to the method described by Pearse (1968), using α-naphthyl acid phosphate as substrate and Fast Garnet GBC as the coupler dye. Blastocysts kept for study of LNAse were first rinsed in 0.1 m cold phosphate buffer, pH 6.5 and stained according to the method of Nachlas et al. (1958) by using L-leucyl-4-methoxy-B-naphthyl-amide-HCl (Sigma) and Fast Blue B salt as coupler. Enzyme activities were analyzed by double-blind observation of vesicle/granule distribution within blastomeres and graded as follows: strong (++++ & ++++) to moderate (+ + & +) to nil (± & -ve). All data were subjected to statistical analysis by applying either proportion(Z) tests or Chi-square test using the Yate's correction factor.

**Table 1.**
Interaction of anti-oestrogen (Nafoxidine-HCl) and oestradiol-17β on the expansion of day 5 early blastocysts of rat in in vitro culture for 12 h.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Experiments</th>
<th>No. of replica</th>
<th>Concentration of drug/vehicle per ml</th>
<th>Total No. of blastocysts cultured</th>
<th>Total No. of expanded blastocysts</th>
<th>% of blastocysts expanded after 12 h culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Saline</td>
<td>8</td>
<td>50 µl</td>
<td>120</td>
<td>120</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>Nafoxidine</td>
<td>4</td>
<td>3 µg</td>
<td>113</td>
<td>22</td>
<td>19.4</td>
</tr>
<tr>
<td>III</td>
<td>Nafoxidine + oestradiol-17β</td>
<td>4</td>
<td>3 µg + 1 µg</td>
<td>127</td>
<td>76</td>
<td>59.8</td>
</tr>
<tr>
<td>IV</td>
<td>Nafoxidine + cholesterol</td>
<td>4</td>
<td>3 µg + 1 µg</td>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>Nafoxidine + ethanol-saline</td>
<td>4</td>
<td>3 µg + 50 µl</td>
<td>45</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Proportion tests between: I and II: P < 0.001, significant. II and III: P < 0.001, significant.
Fig. 1.
Stereomicrograph of day 5 early rat blastocysts before 12 h culture in vitro. × 280.

Results

Expansion of rat blastocysts in vitro

The culture experiments were repeated at least 4 times on different days using a total of 110–130 embryos in each group except for groups IV and V in which a total of 40–45 embryos were employed. Table 1 presents the results of embryo expansion in culture. In group I we found that 100% of the blastocysts expanded after 12 h in culture medium containing only ethanol-saline (Figs. 1 and 2). In the second group, exposure of blastocysts to medium containing nafoxidine caused significant ($P < 0.001$) reduction of expansion (Fig. 3). In the third group addition of oestradiol-17β and nafoxidine to the culture medium allowed blastocysts to expand (Fig. 4). Supplementation with 1 µg cholesterol or 50 µl ethanol-saline along with the anti-oestrogen failed to reverse the inhibitory action of

Fig. 2.
Stereomicrograph of day 5 rat blastocysts after 12 h culture in medium containing ethanol-saline vehicle. Blastocysts show full expansion with large blastocoelic cavities. × 280.
Rat blastocysts after 12 h culture in medium containing nafoxidine. Embryonic cell masses appear crumpled with large perivitelline spaces (→). × 280.

the antagonist on blastocyst expansion. The concentration of 3 μg/ml of nafoxidine was chosen since it was found to be effective in inhibiting blastocyst expansion in vitro and this effect could be successfully reversed by adding oestradiol-17β to the culture medium.

Effects of nafoxidine and oestradiol-17β on activity patterns of ACPase and LNAse in rat blastocysts

Two lysosomal enzyme activities were studied in blastocysts which had expanded in vitro. Table 2 gives the result of ACPase activity found in rat blastocysts. Intense ACPase activity was found in all

the blastomeres while in unexpanded nafoxidine-exposed blastocysts there was significant fall in enzyme activity; ACPase activity was again higher in embryos which had expanded following culture in medium containing the antagonist and oestradiol-17β. Table 3 shows the semiquantitative assessment of LNAase activity in expanded and unexpanded rat blastocysts. In group I, expanded blastocysts exhibited low LNAase activity in comparison to group II blastocysts which showed intense LNAase activity following inhibition of expansion induced by anti-oestrogen. Administration of oestradiol-17β and nafoxidine again caused a fall in the concentration of enzyme-rich granules from the blastomeres.

Discussion

In rodents, expansion of the blastocyst precedes the attachment reaction during which close contact is established between uterine epithelium and trophoblast cells (Nilsson 1967). Biggers et al. (1978) observed that mouse blastocyst expansion occurred as a result of rapid accumulation of fluid and electrolytes due probably to increased membrane permeability to water and ions. During early embryonic differentiation changes have been observed in surface agglutinability (Pienkowski 1974), transport of ions like Na⁺, K⁺ and Ca⁺ (Powers & Tupper 1975) and in membrane potential (Cross et al. 1975) which may lead to changes in the perme-

Table 2.
Interaction of anti-oestrogen (Nafoxidine-HCl) and oestradiol-17β on distribution of acid phosphatase in day 5 blastocysts cultured in vitro for 12 h.

<table>
<thead>
<tr>
<th>Total No. of blastocysts</th>
<th>Control group</th>
<th>Experimental groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(50 µl saline ethanol/ml)</td>
<td>Nafoxidine (3 µg/ml)</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+++++ &amp; +++</td>
<td>16 (35)</td>
<td>3 (11)</td>
</tr>
<tr>
<td>++ &amp; +</td>
<td>28 (62)</td>
<td>21 (77)</td>
</tr>
<tr>
<td>± &amp; -ve</td>
<td>1 (2)</td>
<td>3 (11)</td>
</tr>
</tbody>
</table>

Chi-square tests between: a & b: P < 0.001; a & c: P > 0.05.
Figures in parentheses indicate percentage.

Table 3.
Interaction of anti-oestrogen (Nafoxidine-HCl) and oestradiol-17β on distribution of leucynaphthylamidase in day 5 rat blastocysts cultured in vitro for 12 h.

<table>
<thead>
<tr>
<th>Total No. of blastocysts</th>
<th>Control group</th>
<th>Experimental groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(50 µl saline ethanol/ml)</td>
<td>Nafoxidine (3 µg/ml)</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+++++ &amp; +++</td>
<td>13 (35)</td>
<td>36 (100)</td>
</tr>
<tr>
<td>++ &amp; +</td>
<td>24 (64)</td>
<td>0</td>
</tr>
<tr>
<td>± &amp; -ve</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Chi-square tests between: a & b: P < 0.001; a & c: P > 0.05.
Figures in parentheses indicate percentage.
ability of membranes. It has been reported that pre-implantation rabbit (Dickmann et al. 1975), rat (Dickmann & Dey 1974), hamster (Dickman & Sengupta 1974) and mouse (Dey & Dickmann 1974) embryos have the potential of synthesizing various steroid hormones. It has been hypothesized that production of steroid hormones by the embryos themselves may be necessary for morula-blastocyst transformation and implantation (Dickmann et al. 1976). In the present study we have investigated this problem further to observe whether endogenous oestrogen does indeed affect embryonic maturation and expansion at the blastocyst stage. Oestrogen is known to increase the permeability of cells to water and ions (Spaziani 1975). Our studies have shown that nafoxidine prevents blastocyst expansion in vitro. Nafoxidine is known to bind competitively with oestrogen receptors present in cells and the antagonist can be displaced with increasing concentrations of oestrogen (Katzenellenbogen et al. 1979). In the present study we also find that addition of 1 µg oestradiol-17β to the culture medium containing anti-oestrogen counteracted the anti-oestrogenic response and blastocysts again expanded. To check for the specificity of this response we used cholesterol, another steroid compound, to find if this effect of oestrogen was due to its specific interaction with cellular receptors or was a membrane dependent non-specific response.

Cholesterol is known to have prominent effects on membrane permeability through changes in membrane conformation and enzyme levels (Heiniger et al. 1976). We found, however, that cholesterol is unable to reverse the anti-oestrogenic effect of nafoxidine on blastocyst expansion. We also added ethanol to in vitro culture medium containing nafoxidine and found that it also has no effect on embryo expansion. These studies therefore provide evidence in support of a specific action of anti-oestrogen on blastocoel expansion. Sengupta et al. (1977) have shown that CI-628 citrate, an anti-oestrogen, prevented mouse blastocyst expansion in vitro.

Acid phosphatase, an enzyme found predominantly in the lysosomal fraction is found in pre-implantation embryos of mouse (Solter et al. 1973; Sengupta et al. 1979), rat (Schlafke & Enders 1975; Sengupta et al. 1979) and hamster (Ishida 1972). Ezzel & Szego (1977, 1979) reported that lysosomes may be involved in rat oocyte maturation and germinal vesicle breakdown. In the present study we find changes in ACPase and LNase activity in blastocysts exposed to nafoxidine in the culture medium. There is substantial evidence to support an influence of oestrogenic hormones on lysosomal membrane, and activation of lysosomal enzymes was also observed in oestrogen sensitive tissues, such as the uterus. Pietres & Szego (1979) reported that proteases could be involved in inducing changes in endometrial surface membrane, and that this was stimulated by oestrogen. Guerrier et al. (1977) and Peaucellier (1977) have observed that lysosomal proteases are involved in oocyte maturation in vertebrates. Our studies have shown some changes in the activities of the lysosomal enzymes ACPase and LNase during the time when trophoblastic maturation occurs with changes in membrane structure and permeability. Studies are in progress to investigate changes in membrane specific enzymes during blastocyst maturation and expansion.

Acknowledgments
This work was supported by grants from the Indian Council of Medical Research and from the Family Planning Foundation. We thank the Upjohn Company, USA for their generous gift of Nafoxidine.

References


Received on December 12th, 1980.