Effect of Estradiol-17 β and Progesterone in Co-Culture with Uterine Epithelium Cells on Rat Embryo Development

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Abstract: This experiment was designed to evaluate the ability of Estradiol-17 β (E₂) and progesterone (P₄) to promote development of rat blastocysts. Eight-cell rat embryos were co-cultured for 5 days with rat uterine epithelium (UE) cells with inclusion of E₂ and/or P₄. Embryos were collected from mature Wistar-Imamichi rats. UE cells were recovered from the same washed with modified TCM199 (m 199FCS) and placed in 4-well Multi-dishes. UE cells of monolayers were developed to 70–80% confluence prior to initiation of the embryo culture. Embryos were recovered, selected and randomly placed in one of the four steroid hormone dose concentration media. They were evaluated every 24 h to determine their stage of development. More ($p < 0.05$) embryos were developed to both hatched and post-hatched blastocysts in E₂ with a concentration of 3.5×10^{-5} , 10^{-4} M and P₄ with a concentration of 3.5×10^{-6} , 10^{-5} , 10^{-4} M. The combination of E₂ (3.5×10^{-5} M) and P₄ (3.5×10^{-6} , 10^{-5} , 10^{-4} M) also improved ($p < 0.05$) both hatched and post-hatched blastocysts. These findings showed that the presence of ovarian steroid hormones in a primary culture of UE cells promotes *in vitro* development of post-blastocysts in rat embryos.

Key words: Estradiol-17 β , Progesterone, Co-culture, Rat embryo, Uterine epithelium cell.

The culture of embryos *in vitro* removes the embryo from the maternal environment where it is bathed in an ever-changing natural fluid containing a range of proteins and ions specific to reproductive fluid [1, 2]. In a recent study [3] it was observed that 1-cell rat embryos developed to the blastocyst stage when co-cultured with oviductal cells, so that the use of oviductal cells in embryo co-culture systems has attracted attention in understanding the mechanisms by which helper cells

interact with embryos to enhance *in vitro* development. Development of the preimplantation mammalian embryo *in vitro* occurs in a complex luminal environment whose volume and composition are under the control of the ovarian hormones progesterone (P₄) and estradiol-17 β (E₂) [4, 5]. It has been proposed that interaction between uterine epithelial (UE) cells and various regulatory agents (hormones and growth factors) effect (induce, stimulate or repress) structural and functional changes at the apical surface of the UE cell that allow nidation. Nevertheless, the UE cellular mechanisms and the effects on embryos involved in these P₄: E₂ regulated processes are not clearly understood.

Receptivity is therefore not accomplished by a simple "turn-on" of protein or glycoprotein synthesis [6, 7], but rather regulatory complexes acting directly on the UE cell paracrine factors may coordinately and differentially vary molecular synthesis and traffic to the appropriate plasma membrane domain of the UE cell.

The object of this study was to evaluate the effect of ovarian hormones in co-culture with uterine epithelium cells on 8-cell rat embryo development.

Materials and Methods

Animals and Embryo collection: Adult virgin female rats of Wistar-Imamichi strain weighing from 200 to 240 g were used. They were maintained under controlled light conditions (12 h light: 12 h darkness: light on at 6:00 h) and allowed to take pelleted diet and water freely. Vaginal smears were taken in the morning for assessment of proestrus and graded. They were mated with the same strain fertile males on the evening of proestrus. The day when spermatozoa were detected in the vaginal smear was considered day 1 of pregnancy. Eight-cell embryos were collected by flushing the oviduct with culture medium in the early morning of day 4.

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Collection of uterine epithelial cells: The uteri were trimmed free of ligaments and slit lengthwise on the side of the mesosulphinx. Cells from the uterus were collected by lightly scraping the luminal surface with a scalpel and suspended in tissue culture medium in a 4-well Multi-dish (Nunc, Denmark). The UE cells were cultured at 37.5°C in an atmosphere of 95% air: 5% CO₂. UE cell monolayers were developed for 4 days to 70–80% confluence before starting the embryo culture.

Culture medium and culture: The basic culture medium was modified Krebs-Ringer-bicarbonate solution [8] containing 25 mM lactate, 0.25 mM pyruvate and supplemented with antibiotics and 1 mg/ml bovine serum albumin. Embryos were randomly assigned to each treatment group from the pooled embryos. All incubations were carried out at 37.5°C and in a humidified atmosphere of 5% CO₂ and 95% air. The medium was changed 48 h after starting the culture. For co-culture, TCM199 (Sigma, USA) was supplemented with sodium lactate 25 mM, sodium pyruvate 0.5 mM and 10% fetal calf serum (modified 199; m199FCS). Penicillin (20 IU/ml) and streptomycin (20 µg/ml) were also added as antibiotics. According to the experimental design P₄ and E₂ (Sigma, USA) were added to the culture medium in different concentrations as follows:

Experiment 1: Only P₄ in concentrations of 3.5×10^{-6} , 3×10^{-5} and 3×10^{-4} M.

Experiment 2: Only E₂ in concentrations of 3.5×10^{-6} , 3×10^{-5} , and 3×10^{-4} M.

Experiment 3: Co-culture with P₄ and E₂ in a combination. P₄ are 3.5×10^{-6} , 10^{-5} and 10^{-4} M and E₂ 3.5×10^{-5} M.

Experiment 4: In conditioned medium, P₄ and E₂ were added in the same concentrations as in Experiment 3. For this experiment, UE monolayers were developed for 4 days to 70–80% confluence and the supernatant were collect and used as conditioned medium.

All these doses of steroid hormone mentioned above cover the physiological range of concentrations.

Evaluation of embryo development and statistical analysis: Post-hatching embryos were recorded as achieving stage 1 when they displayed proliferating trophoectoderm cells and stage 2 when they showed outgrowth of trophoectoderm cells on the bottom of the culture vessel. Data were evaluated for statistically significant differences by χ^2 -test.

Results

Effects of the inclusion of P₄ in co-culture on the development of rat embryos are shown in Table 1. There was no difference between the control and P₄ treatment groups in terms of blastocyst development, but both hatched and stage 1 post-hatched blastocysts increased significantly ($p < 0.05$) compared with the control. No difference among the P₄ doses was found, but the concentrations of 3.5×10^{-6} and 3×10^{-5} M of P₄ resulted in better post-hatched development.

Effects of the inclusion of E₂ in co-culture on the development of rat embryos are shown in Table 2. Like P₄, there was no difference between the control and E₂ treatment groups in terms of blastocysts. Here, hatched and post-hatched blastocyst development at both stages increased significantly ($p < 0.05$) with the 3.5×10^{-5} and 3×10^{-4} M concentrations of E₂ compared with the control. Combined effects of P₄ (3.5×10^{-6} , 10^{-5} , 10^{-4} M) and E₂ (3.5×10^{-5} M) on the development of rat embryos cultured in m199 FCS medium are shown in Table 3. Development remains similar up to the blastocyst stage but the hatched and post-hatched blastocyst developments in both stages increased significantly ($p < 0.05$) in all concentration groups other than the control.

Similarly, when the P₄ concentration was to 3.5×10^{-6} , 10^{-5} , 10^{-4} M with E₂ (3.5×10^{-5} M) in the conditioned

Table 1. Development of rat embryos in co-culture: effect of the inclusion of progesterone in the m199 FCS medium

Day of culture	Developmental stage	Progesterone ($3.5 \times M$)			
		0	10^{-6}	10^{-5}	10^{-4}
0	Eight-cell	323 (%)	215 (%)	210 (%)	203 (%)
1	Morula	310 (96.0) ^a	207 (96.3) ^a	202 (96.2) ^a	194 (95.6) ^a
2	Blastocyst	303 (93.8) ^a	203 (94.4) ^a	197 (93.8) ^a	190 (93.6) ^a
3	Hatched blastocyst	61 (18.9) ^a	76 (35.3) ^b	71 (33.8) ^b	61 (30.0) ^b
4	Post-hatch stage 1	35 (10.8) ^a	65 (30.2) ^b	62 (29.5) ^b	56 (27.6) ^b
5	Post-hatch stage 2	33 (10.2) ^a	65 (30.2) ^b	61 (29.0) ^b	52 (25.6) ^b

^{a, b} differ significantly from each other ($p < 0.05$).

medium, results followed the same trend as in Tables 3 and 4. Development remains similar up to the blastocyst stage but the hatched and post-hatched blastocyst development of both types increased significantly ($p < 0.05$) in all combination groups other than the control.

Discussion

The addition of P_4 and/or E_2 has shown effects on hatched blastocysts and post-hatched blastocysts in co-

culture with UE cells.

The secretions of UE support survival of both the sperm and the blastocysts [9, 10]. The UE cells provide an apical surface, which permits embryo attachment [11–13]. There was also observed a basal surface that presumably transduces a blastocyst signal, which initiates stromal cell differentiation [14–16]. The UE cell responses can be directed by alterations in the hormonal environment [17–20], and it is likely that expression of those components which are directly in-

Table 2. Development of rat embryos in co-culture: effect of the inclusion of estradiol-17 β in m199 FCS medium

Day of culture	Developmental stage	Estradiol-17 β ($3.5 \times M$)			
		0	10^{-6}	10^{-5}	10^{-4}
0	Eight-cell	323 (%)	218 (%)	215 (%)	209 (%)
1	Morula	310 (96.0) ^a	210 (96.3) ^a	206 (95.8) ^a	200 (95.7) ^a
2	Blastocyst	301 (93.2) ^a	205 (94.0) ^a	205 (95.3) ^a	198 (94.7) ^a
3	Hatched blastocyst	61 (18.9) ^a	44 (20.2) ^a	55 (25.6) ^b	54 (25.8) ^b
4	Post-hatch stage 1	35 (10.8) ^a	24 (11.0) ^a	40 (18.6) ^b	37 (17.7) ^b
5	Post-hatch stage 2	33 (10.2) ^a	23 (10.6) ^a	38 (17.7) ^b	32 (15.3) ^b

^{a, b} differ significantly from each other ($p < 0.05$).

Table 3. Development of rat embryos in m199 FCS medium with addition of progesterone (3.5×10^{-6} , 10^{-5} , 10^{-4}) and estradiol-17 β (3.5×10^{-5} M)

Day of culture	Developmental stage	Co-culture with progesterone ($3.5 \times M$)			
		0	10^{-6}	10^{-5}	10^{-4}
0	Eight-cell	323 (%)	209 (%)	201 (%)	213 (%)
1	Morula	310 (96.0) ^a	202 (96.7) ^a	201 (100.0) ^a	210 (98.6) ^a
2	Blastocyst	303 (93.8) ^a	196 (93.8) ^a	194 (96.5) ^a	203 (95.3) ^a
3	Hatched blastocyst	61 (18.9) ^a	115 (55.0) ^b	131 (65.2) ^c	124 (58.2) ^b
4	Post-hatch stage 1	35 (10.8) ^a	71 (34.0) ^b	84 (41.8) ^c	64 (30.0) ^b
5	Post-hatch stage 2	33 (10.2) ^a	61 (29.2) ^b	79 (39.3) ^c	61 (28.6) ^b

^{a, b, c} differ significantly from each other ($p < 0.05$).

Table 4. Development of rat embryos in conditioned medium with the addition of progesterone (3.5×10^{-6} , 10^{-5} , 10^{-4}) and estradiol-17 β (3.5×10^{-5} M)

Day of culture	Developmental stage	Conditioned medium with progesterone ($3.5 \times M$)			
		0	10^{-6}	10^{-5}	10^{-4}
0	Eight-cell	218 (%)	211 (%)	204 (%)	207 (%)
1	Morula	208 (95.4) ^a	202 (95.7) ^a	202 (99.0) ^a	197 (95.2) ^a
2	Blastocyst	200 (91.7) ^a	195 (92.4) ^b	192 (94.1) ^b	194 (93.7) ^b
3	Hatched blastocyst	36 (16.5) ^a	115 (54.5) ^b	129 (63.2) ^b	118 (57.0) ^b
4	Post-hatch stage 1	19 (8.7) ^a	67 (31.8) ^b	83 (40.7) ^c	61 (29.5) ^b
5	Post-hatch stage 2	19 (8.7) ^a	46 (21.8) ^b	78 (38.2) ^c	57 (27.5) ^b

^{a, b, c} differ significantly from each other ($p < 0.05$).

volved in achieving specialized UE cell roles is hormonally regulated. Delivery to and maintenance of such components at the appropriate site of function must be dependent on both hormone and epithelial cell polarity [21–23].

Blastocyst development remains the same in both P_4 and E_2 , but hatching and post hatching increased with P_4 although no direct comparison was made between them. It is also interesting that the combination of P_4 and E_2 is more effective. Little is known about the control of growth and differentiation of preimplantation embryos. Two distinct features during preimplantation embryo development are activation and cleavage of the embryo following fertilization and differentiation of embryonic cells into an inner cell mass and trophoectoderm at the blastocyst stage. In the mouse or rat, P_4 and E_2 are required for normal preimplantation embryo development in the reproductive tract [24, 25]. Although fertilized embryos can develop into blastocysts within the reproductive tract of ovariectomized animals, a substantial loss in the number of embryos and a decrease in the number of cells per embryo occur. Treatment of these animals with an appropriate combination of P_4 and E_2 corrects these defects [24, 25]. These observations suggest that while certain growth-promoting factors originating in the embryo are involved in autocrine regulation of embryo growth and differentiation, the full complement of preimplantation embryo development and differentiation requires additional paracrine factors that originate in the reproductive tract under the influence of P_4 and/or E_2 . No direct effects of P_4 and/or E_2 on preimplantation embryo development have been documented.

Furthermore, blastocysts developed from 8-cell embryos cultured in groups, or singly in the presence of epidermal growth factor (EGF), showed a higher incidence of zona-hatching compared with those cultured in the absence of EGF [26]. The detection of EGF receptors on the embryonic cell surface at the 8-cell/morula and blastocyst stages supports our contention that EGF or TGF- α can influence preimplantation embryo development and blastocyst functions. The effects of EGF or TGFs are specific, since insulin-like growth factor-I (IGF-I) had no effect on embryo development [26].

The combination of P_4 and E_2 is more effective for hatching. It is possible to speculate that EGF production may be high in this condition. EGF has been linked with UE cell proliferation [27, 28]. Estrogen behaves as a mitogen by inducing increases in EGF messenger RNA before the appearance of the functional receptor,

which precedes DNA synthesis [27, 29]. Estrogen can increase the level of functional EGF *in vivo* [27, 30], promoting, via its specific tyrosine protein kinase cascade, the stimulation of transcription and the synthesis of specific proteins involved in cell division. Julian *et al.* [31] reported that, in defined medium, which contained E_2 ($2.5 \times 10^{-9}M$), UE cells that proliferated to confluence demonstrated separation of apical and basal plasma membrane domains and displayed preferential secretion of proteins and proteoglycans from the apical surface.

One of the major roles of P_4 in the estrus cycle is to prepare the uterus for implantation. The implantation process is a complex interaction between embryonic and uterine cells and cell type-specific interactions between P_4 and E_2 , but the monocular and cellular mechanisms involved in these P_4 : E_2 regulated processes are not clearly understood. One emerging concept is that the P_4 : E_2 effect in the uterus and/or the embryos is transduced by growth factors, which function in an autocrine/paracrine manner.

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