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The influence of endometrium on morphology and oestrogen release of Day-11 and Day-13 pig blastocysts *in vitro*

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Abstract

Day-11 spherical and Day-13 filamentous blastocysts were cultured *in vitro* without supplementation of oestrogen precursors for 2 consecutive periods of 24 h, with or without endometrial tissue. Spherical Day-11 blastocysts flushed from one uterine horn (5.5 ± 0.8 blastocysts; 5.3 ± 0.4 mm in diameter; mean ± s.d.) did not show gross morphological changes during the culture period. Free and conjugated oestrone (E1) and oestradiol-17β (E2) were released during the first and second 24 h culture period and was maximal during the first 24 h. In the presence of endometrial tissue the release of oestrogens from the blastocysts was reduced to 30-50%.

Clewed Day-13 filamentous blastocysts, recovered after flushing, formed trophospheres and also in these cultures both free and conjugated E1 and E2 were released. In this case the addition of endometrial tissue did not affect the release of oestrogens from the blastocysts. Single Day-13 filamentous blastocysts cultured without endometrial tissue (n = 33, from 4 gilts), developed trophospheres and all blastocysts released E1 and E2. During the first 24 h on average per blastocyst 2.6 ng E1 (range 1.6–4.0) and 9.7 ng E2 (range 4.3–15.3) were released. In all cultures of spherical and filamentous blastocysts E2 release exceeded E1 release.

These data show that Day 11–13 blastocysts are able to release both free and conjugated E1 and E2 during *in vitro* culture from endogenous precursors. The addition of endometrial tissue reduced the release of oestrogens from Day-11 but not Day-13 blastocysts. Whether this is due to an altered oestrogen release of the blastocysts in the presence of endometrium on Day-11 or due to an increased uptake of oestrogens by the endometrium on Day-11 remains to be investigated.

Keywords: oestrogen, *in vitro* culture, pig, blastocyst, endometrium

Introduction

In the pig oestrogens synthesized and released by the blastocysts are assumed to be responsible for maternal recognition of pregnancy, by changing the direction of endometrial $PGF_{2\alpha}$ towards the uterine lumen (Bazer & Thatcher, 1977). Oestrogens can also induce the release of other substances from the endometrium to the uterine lumen such as calcium and uterine proteins (Geisert et al., 1982; Geisert et al., 1987). It is therefore obvious that blastocysts, by means of secreting oestrogens, can influence the secretion of the endometrium during Day 10–13 of pregnancy. In our concept the interaction between the blastocysts and the endometrium is a dynamic process. The endometrium or its secretion products may therefore affect the amount or type of oestrogen secretion from the blastocysts or may induce ultrastructural changes in the blastocyst such as elongation. Until now no evidence has been provided that oestrogens by themselves can induce elongation in pig blastocysts (O'Neill et al., 1991). This suggests a role for the endometrium or its secretion products in the process of elongation.

The object of this study was to investigate whether co-culture of endometrium and embryo's could influence each other on aspects of morphology and secretory capacity.

We therefore studied the release of free and conjugated oestrogens by Day-11 spherical and Day-13 filamentous blastocysts, during *in vitro* culture with or without endometrial tissue using a medium without supplementation of potential growth factors or precursors of steroids. The morphological features of blastocysts and uterine luminal epithelium were examined after culture.

Materials and methods

Animals. Crossbred gilts (Great Yorkshire × Dutch Landrace), which had shown 2 or more normal estrous cycles (18–22 days), were checked for estrous with a vasectomized boar twice daily (09:00 and 15:00 h). At the time of observing first standing oestrus (Day 0) 100 µg GnRH analogue (Ovalyse: Upjohn Company, Ede, The Netherlands) were injected i.m. and the gilts were artificially inseminated on Day 1, 24 h after GnRH injection (Van der Meulen et al., 1986).

The gilts were slaughtered on Day 11 or 13 of pregnancy. Within 10 min after stunning and exsanguination the blastocysts were recovered by flushing the left uterine horn twice with 30 ml Dulbecco's phosphate-buffered saline (PBS: Gibco, Paisley, Scotland). The other horn was not used in this experiment. The diameters of the spherical Day-11 blastocysts were measured. On Day 13 a clew of filamentous blastocysts was recovered upon flushing. From the middle of the uterine horn small pieces of endometrial tissue were dissected for co-cultures with endometrium. The blastocysts and endometrial tissue were transferred to the laboratory within half an hour in Minimal Essential Medium^3 (MEM^3: Gibco) at a constant temperature of 37 °C.

To collect individual filamentous blastocysts, the uterine horns of 4 Day-13 gilts were transported on ice to the laboratory. The mesometrium was detached and one horn was cut longitudinally along the antimesometrial side. The opened horn was

placed in a dissection tray in Dulbecco's PBS and pinned in stretched position to the wax base.

The blastocysts were detached from the endometrium by a gentle stream of buffer after stretching of the endometrial folds (Bate & King, 1988).

Culture. The spherical Day-11 blastocysts recovered from one horn were cultured together with (n = 7) or without (n = 7) small pieces of endometrial tissue, just as the Day-13 clewed filamentous blastocysts (with endometrial tissue, n = 5; without endometrial tissue, n = 6). Filamentous blastocysts collected by opening one uterine horn of each of 4 gilts were cultured individually (n = 33) without endometrial tissue.

All cultures were carried out for 48 h in 4 ml MEM[4] (Gibco), supplemented with penicillin (100 IU ml^{-1}: Sigma, St Louis, MO, USA), streptomycin (400 µg ml^{-1}: Sigma) and 1% non essential amino acids (NEAA: Gibco) at 37 °C in 5% CO_2 in air for 48 h. After the first 24 h the medium was renewed.

Electron microscopy. Endometrial tissue at the onset of culture and after 48 h of co-culture was processed for transmission electron microscopy (TEM: Stroband et al., 1986). The ultrastructure of the uterine luminal epithelium upon culture was compared with the *in vivo* morphology of uterine luminal epithelium during Day 11–13 and Day 13–15 respectively. After 48 h of culture the blastocysts were processed for light microscopy.

Oestrogen assay. In each culture total and free E1 and E2 were measured after extraction and column chromatography by RIA, according the methods described by Fischer et al. (1985) and Helmond et al. (1980). Two samples (100 µl) of each culture were diluted in 900 µl phosphate buffer BSA with 0.1% BSA and mixed with 1 ml acetate buffer (0.15 M, pH 4.1). To determine the total oestrogen content, conjugated oestrogens were converted to free oestrogens, by incubating one of the two samples overnight at 37 °C in the dark after addition of an enzyme preparation, containing sulphatase (3100 units ml^{-1}) and ß-glucuronidase (130000 units ml^{-1}) from the snail *Helix pomatia* (Sigma). Both samples were extracted 3 times with diethyl ether (4 ml) after the addition of 1000 cpm of [2,4,6,7-^{3}H]E1 and [2,4,6,7,16,17-^{3}H]E2 (Radiochemical Centre, Amersham, UK) for estimation of procedural losses. The residues of the diethyl ether extracts were dried under a stream of nitrogen, redissolved in 250 µl toluene:methanol (9:1, v/v) and applied to chromatography columns (8.0 × 0.7 cm) packed with Sephadex LH-20 (Pharmacia, Uppsala, Sweden; eluting solvent: toluene:methanol 9:1, v/v). The first 1 ml fraction was discarded, E1 was eluted in the next 3 ml fraction and after discarding another 0.5 ml fraction E2 was eluted in a final 3 ml. The E1 and E2 fractions were dried under a stream of nitrogen and redissolved in 500 µl ethanol. An aliquot of 150 µl was taken in order to determine the recovery of [^{3}H]E1 (78%) and [3H]E2 (72%), respectively.

E1 concentration was measured in duplicate (2 aliquots of 150 µl) by RIA using a specific rabbit antiserum against 6-keto-estrone 6-CMO-BSA. The main cross-reacting steroid was oestradiol (0.45%). The antiserum was used in a working dilution of

1:25000. The sensitivity of the assay was 13 pg ml^{-1} at the 90% B/B0 level. The intra-assay coefficient of variation was 12.4% and the inter-assay coefficient of variation was 17.4%.

E2 concentration was measured in duplicate (2 aliquots of 150 µl) by RIA using a specific rabbit antiserum against 6-keto-17ß-oestradiol 6-CMO-BSA. The main cross-reacting steroids were estrone (1.49%) and estriol (0.21%). The antiserum was used in a working dilution of 1:30000. The sensitivity of the assay was 14 pg ml^{-1} at the 90% B/B0 level. The intra-assay coefficient of variation was 11.5% and the inter-assay coefficient of variation was 16.5%. The amount of E1 and E2 are expressed in ng 24 h^{-1} after correction for procedural losses.

Statistical analysis. After ln transformation of the data differences in free and total E1 and E2 release between cultures were tested for significance by analysis of variance (SPSS Inc, 1988). Data are expressed as mean ± s.d.

Results

On Day 11, 5.5 ± 3.1 spherical blastocysts with a diameter of 5.3 ± 1.4 mm were recovered per uterine horn. The same number of blastocysts, with equal sizes, were used in cultures with and without endometrial tissue. During culture no change in the diameter of the spherical blastocysts was observed and the blastocysts preserved their morphology. At the onset of co-culture of Day-11 blastocysts and endometrial tissue, uterine epithelial cells showed an accumulation of secretory vesicles at the luminal plasma membrane. This ultrastructural feature did not change during the 48 h *in vitro* culture period (Figures 3 and 4).

During culture with and without endometrial tissue the clewed Day-13 filamentous blastocysts formed numerous translucent trophospheres along the length of the trophoblast (Figure 1). The trophospheres were composed of flat trophoblast cells and the inside was furnished with a network of hypoblast cells (Figure 2). Trophospheres also developed along filamentous blastocysts collected and cultured individually. At the onset of co-culture of Day-13 filamentous blastocysts and endometrial tissue, uterine luminal epithelial cells showed a concentration of small dark granules at the basal side of the cells. These granules were also found after 48 h of culture (Figure 5).

In cultures of Day-11 blastocysts free and conjugated E1 and E2 were released (Table 1), but this release was significantly lower during the second 24 h culture period ($P<0.05$). Release of free E1 and E2 was significantly larger during both culture periods for cultures without endometrial tissue ($P<0.01$). The release of free and total E2 was significantly larger than the release of respectively free and total E1 ($P<0.05$).

In cultures of Day-13 clewed filamentous blastocysts free and conjugated E1 and E2 were released during both culture periods and there were no significant differences between cultures with and without endometrial tissue (Table 1). Oestrogen release did not differ significantly for the first and second 24 h of culture. During both culture periods significantly more E2 than E1 was released ($P<0.05$).

Table 1. The release of free and total E1 and E2 by spherical Day-11 and filamentous clewed Day-13 blastocysts flushed from one uterine horn, during *in vitro* culture with or without endometrial tissue (end) for 2 periods of 24 h (mean and range).

Day	End	Culture (h)	n	E1 (ng per 24 h)		E2 (ng per 24 h)	
				free	total	free	total
11	+	0–24	7	1.9	4.1	9.0	12.1
				0.4–3.2	0.5–8.8	0.4–22.5	0.7–31.4
		24–48		0.9	2.3	2.7	3.9
				0.1–1.6	0.1–6.5	0.1–6.9	0.1–12.9
11	–	0–24	7	7.2	9.2	34.2	41.2
				1.1–15.8	1.6–19.9	16.0–61.8	18.2–92.3
		24–48		2.9	3.6	14.5	18.7
				0.7–6.5	2.4–6.9	1.0–33.0	3.4–47.7
13	+	0–24	5	2.6	4.6	29.1	29.0
				1.9–4.2	2.9–9.0	20.0–44.0	9.2–58.8
		24–48		4.0	6.4	21.0	28.9
				2.9–5.6	4.0–8.6	6.5–39.6	9.2–48.1
13	–	0–24	6	6.7	7.5	32.2	44.7
				2.3–11.5	3.2–11.6	7.3–61.8	12.2–92.9
		24–48		4.8	5.5	17.2	21.9
				0.9–7.9	3.3–8.0	3.1–36.8	4.0–37.7

Table 2. The average release of free and total E1 and E2 by individual Day-13 filamentous blastocysts collected by opening one uterine horn of each of 4 gilts, during *in vitro* culture for 2 periods of 24 h.

Gilt	n	E1 (ng per 24 h per blastocyst)		E2 (ng per 24 h per blastocyst)	
		free	total	free	total
		culture period 0–24 h			
125	6	4.0^a	4.2^a	15.3^a	19.3^a
133	8	2.2	2.6^b	14.3^a	20.4^a
315	10	2.9^a	3.0	7.7^b	26.1^a
11	9	1.6^b	3.8	4.3^b	4.9^b
mean	33	2.6	3.3	9.7	17.7
		culture period 24–48 h			
125	6	3.0^a	3.2^a	9.6^b	10.8^c
133	8	3.7^a	3.8^a	19.5^c	27.3^d
315	10	1.0^b	1.4^b	3.2^a	4.6^a
11	9	0.9^b	1.7^b	2.3^a	2.7^b
mean	33	2.0	2.4	8.0	10.7

Column means per culture period with a different superscript differ significantly ($P<0.05$)

endometrial tissue in culture. Uterine epithelial cells at Day 13 contain dark granules at the bases of the cells, these granules were also observed in uterine epithelial cells after 48 h of *in vitro* culture. In endometrial tissue dissected from Day-15 pregnant gilts these granules are not observed (Stroband et al., 1986).

In spite of retarded development of both embryos and endometrium during culture, analysis of the culture media shows secretion of oestrogens. Both Day-11 spherical and Day-13 filamentous blastocysts release E1 and E2. The addition of oestrogen precursors to the culture medium is apparently not necessary for this release. This observation is in accordance with oestrogen release by Day-16 trophoblast explants during *in vitro* culture in absence of oestrogen precursors (Heap et al., 1980). The decrease in oestrogen release during the second culture period of 24 h of Day-11 blastocysts corresponds with the decrease in oestrogen release by Day-16 trophoblast explants (Heap et al., 1980). The results of the present study indicate that both Day-11 and Day-13 blastocysts possess endogenous precursors which are converted to oestrogens. Decrease in oestrogen release during the second culture period, except in Day-13 co-culture, may be caused by a deprivation of endogenous precursors. Decreased oestrogen release may also be caused by loss of enzyme activity, since e.g. aromatase activity is decreased on Days 3.5–7 compared with Days 1–3.5 in cultures of dispersed Day-15 blastocysts (Whyte et al., 1986-1987).

In the present study a significantly larger release of E2 compared with E1 has been measured. In most studies (Perry et al., 1973, 1976; Gadsby et al., 1980; Whyte et al., 1986–1987) incubations of blastocysts with labelled precursors yielded a larger conversion of E1 compared with E2. Incubation conditions may have been decisive in these studies. Incubation with a NADPH-generating system (Van der Meulen et al., 1989) resulted in a (10-fold) larger conversion of labelled androstenedione to E2 compared with E1 by homogenized filamentous Day-13 blastocysts (J. van der Meulen, G. te Kronnie, R. van Deursen and J. Geelen, unpublished data). In uterine flushings from pregnant gilts on Days 10–13 also more E2 than E1 is measured (Zavy et al., 1980; Geisert et al., 1982).

The observed differences between total and free oestrogens indicate the presence of conjugated oestrogens in the medium after culture with and without endometrial tissue. Conjugated oestrogens in uterine flushings are generally attributed to endometrial sulfation of blastocyst free oestrogens (Geisert et al., 1982) by sulfotransferase activity (Pack & Brooks, 1974). Only in a few studies concerning the synthesis of oestrogens from labelled precursors *in vitro*, attention has been paid to conjugated oestrogens. After 3 h incubation of trophoblast tissue of Day 14–18 blastocysts Gadsby et al. (1980) recovered 14.8, 20.3, and 14.9% of the initial amount of respectively labelled testosterone, dehydroepiandrosterone and androstenedione in the aquaous fraction containing e.g. steroid conjugates, sulphates and glucuronides. Conversion of labelled androstenedione to conjugated oestrogens has also been measured in cultures of trophospheres of dispersed Day 14–18 blastocysts (Whyte et al., 1986–1987), and conjugated estrone has been detected in medium after incubation of Day 13–25 embryonal membranes (Guthrie & Lewis, 1986). Therefore, *in vitro* blastocysts seem to be able to conjugate oestrogens, even in cultures without addition of precursors. On Day 11 but not Day 13 the amount of oestrogens recovered in the medium was signif-

icantly decreased by the presence of endometrial tissue. Fischer et al., 1985 showed a similar negative effect of the coincubation of endometrium tissue with Day-16 and Day-25 conceptus material on oestrogen production from labelled progesterone precursors. Whether this phenomenon reflects an altered oestrogen release by the blastocysts or an increased uptake by the endometrium remains to be investigated. An other suggestion is that Day-11 endometrium secretes products that inhibit synthesis or release of estrogens from Day-11 blastocysts.

Oestrogen release by individual blastocysts differs between gilts. Differences in size and concomitant developmental stage, as observed for Day-14 blastocysts (Bate & King, 1988), may partly be responsible for such differences in oestrogen release between gilts.

The amount of oestrogen released in cultures without endometrial tissue by Day-11 and Day-13 flushed blastocysts is almost equal. Oestrogen release by individually recovered Day-13 blastocysts is about a third of the oestrogen release by all blastocysts together, flushed out of one uterine horn. The oestrogen release by Day-13 flushed blastocysts therefore seems relatively low, which may be caused by the diminished surface as a consequence of clewing of the blastocysts. The oestrogen release by individually cultured Day-13 blastocysts suggests that from Day 11 to 13 oestrogen release increases, as may be expected since an increase in oestrogen levels in uterine flushings from Day 10 to 13 has been described (Zavy et al., 1980; Geisert et al., 1982).

The data of this study show that Day 11–13 blastocysts release both free and smaller amounts of conjugated E1 and E2 during *in vitro* cultures. Since no precursors were added to the medium, it is suggested that blastocysts can also synthesize (conjugated) oestrogens from endogenous precursors during this stage of development. This release of oestrogens was however not accompanied by development of blastocysts and endometrium. Thus Day-11 blastocysts did not show elongation even in the presence of endometrium. Endometrium development may be dependent on (systemic) factors which are not present in the co-culture conditions used in this study. Under better conditions the endometrium may release its vesicles which may influence positively aspects of embryo development such as elongation. Close contact between the blastocyst and endometrial tissue, as in the *in vivo situation*, may also be essential for the interaction between blastocyst and endometrium.

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