Accumulation of oestrone by pig blastocysts and its potential physiological significance for blastocyst development *in vitro*

H. Niemann and F. Elsaesser

*Institut für Tierzucht und Tierverhalten (FAL), Mariensee, 3057 Neustadt 1,
Federal Republic of Germany*

Summary. Oestrone accumulation of Day-5 pig blastocysts and the potential physiological significance of oestrone and oestradiol-17 β for blastocyst development were investigated *in vitro*. After 6 h of in-vitro culture in medium supplemented with 10 nM-[³H]oestrone, the accumulation amounted to 550 ± 49 d.p.m. (s.e.m.) per 10 blastocysts. The accumulation of [³H]oestrone (or its metabolite(s)) was reduced ($P < 0.001$) in the presence of a 100-fold excess of unlabelled oestrone or oestradiol-17 β to 135 ± 14 d.p.m. or 148 ± 28 d.p.m. per 10 blastocysts, respectively. The accumulation of [³H]oestrone was not affected in the presence of a 100-fold excess of unlabelled progesterone, testosterone or oestrone sulphate. When blastocysts were post-incubated for 30 or 60 min in [³H]oestrone-free medium, blastocysts retained $74.1 \pm 16.8\%$ and $66.0 \pm 10.4\%$, respectively of their initial radioactivity. In parallel experiments with [³H]progesterone the respective values were $23.8 \pm 3.0\%$ and $21.7 \pm 2.1\%$. The presence of the antioestrogen nafoxidine (15 μ g/ml) in basic culture medium impaired ($P < 0.001$) the transformation of morulae to blastocysts ($21.5 \pm 8.9\%$) compared to controls ($98.3 \pm 1.7\%$). The inhibitory effects could be overcome ($P < 0.001$) by a supplementation with 1 nM- or 100 nM-oestradiol-17 β ($62.5 \pm 12.8\%$ and $80.0 \pm 6.2\%$ development to blastocysts) but not with 1 nM- or 100 nM-oestrone ($30.3 \pm 9.6\%$ and $45.2 \pm 10.5\%$). Blastocyst expansion was also decreased $P < 0.01$ to $61.0 \pm 11.4\%$ of control values in the presence of 15 μ g nafoxidine ml. Oestradiol-17 β (100 nM) reversed the nafoxidine-induced inhibition of blastocyst expansion to $85.5 \pm 5.2\%$ of control values ($P > 0.05$). It is concluded (1) that early pig embryos possess specific binding sites for oestrogens, (2) that oestradiol-17 β is a potentially important factor in morula–blastocyst transformation, and (3) that blastocyst expansion is more independent of oestrogen.

Introduction

Investigations into the endocrine control of early embryonic development in pigs have demonstrated accumulation of both oestradiol-17 β and progesterone by 5-day-old pig blastocysts *in vitro*. The uptake of oestradiol-17 β was associated with the viability of blastocysts and was independent of protein content of the surrounding medium (Niemann & Elsaesser, 1984). Oestradiol-17 β appears necessary for a regular morula–blastocyst transformation and this effect seems to be mediated through specific binding sites (Niemann & Elsaesser, 1986). Whether zona pellucida-intact embryos synthesize oestradiol-17 β themselves, or whether they depend on oestradiol-17 β in their environment, has not been clarified unequivocally (see Niemann & Elsaesser, 1986).

Oestrogens other than oestradiol-17 β may also be involved in early embryonic development. Oestrone occurs at high levels in uterine fluid of pigs on Day 6 of gestation (Zavy *et al.*, 1980) and in trophectoderm and yolk sac endoderm of embryos on Day 10 of gestation (King & Ackerley, 1985).

Furthermore, oestrone is a major oestrogen synthesized by pig blastocysts on Day 12 of pregnancy, when embryonic synthesis of oestrogens appears important in the maternal recognition of pregnancy (Perry *et al.*, 1973; Heap *et al.*, 1979). The present study (1) determines the kind of oestrone accumulation by pig blastocysts and (2) investigates whether oestrone or oestradiol-17 β are potentially more important for morula-blastocyst transformation and subsequent development of blastocysts.

Materials and Methods

Animals and embryos. Superovulation was induced in 105 prepubertal German Landrace gilts with 1500 i.u. PMSG (Seragon: Ferring, Kiel, F.R.G.) followed 72 h later by 500 i.u. hCG (Ekluton: Vemie, Kempen, F.R.G.). Viable embryos were collected from 85 gilts (93.5 ± 12.7 kg body weight, 179 ± 21.3 days of age; mean $\pm$ s.d.) at slaughter, 4 days after mating (Niemann & Elsaesser, 1986). Lack of embryo recovery from the remaining 20 pigs was attributed to ovulation- or fertilization failure. A mean number of 30.9 ± 14.6 corpora lutea was counted in these gilts, yielding a total of 2210 ova and embryos (26.0 ± 13.2 per gilt). Recovery and fertilization rates were $84.3 \pm 14.4\%$ and $91.0 \pm 11.6\%$, respectively. After rejection of retarded or degenerated embryos and unfertilized ova 1723 morphologically intact morula and blastocyst stages were available for experiments. Embryos were cultured at 37°C in an environment of 5% CO_2 in air, in a culture medium based on Krebs-Ringer-Bicarbonate (KRB) solution without bovine serum albumin (BSA) but supplemented with 10% heat-inactivated lamb serum. Embryonic development was observed at 100-fold microscopical magnification and assessed by morphological criteria (Niemann *et al.*, 1983).

Experiment 1: accumulation of [^3H]oestrone. Altogether 420 morulae were cultured in the basic culture medium, changed twice during a 24-h period. Blastocysts were then randomly assigned to groups of 10 and further incubated in 2 ml culture medium supplemented with 10 nM-[2,4,6,7- ^3H]oestrone ($\sim 2\,000\,000$ d.p.m./ml: NET 319, New England Nuclear, Boston, MA, U.S.A., sp. act. 88.5 Ci/mmol , radiochemical purity $>98\%$) for 6 h. The specificity of oestrone accumulation was tested in parallel by incubations with 10 nM-[^3H]oestrone plus a 100-fold excess (1 μM) of unlabelled oestrone, oestradiol-17 β , progesterone, testosterone or oestrone sulphate. After each incubation, embryos were washed 5 times in KRB-solution and transferred into vials containing 0.2 ml tissue solvent (Soluene: Packard, Downers Grove, IL, U.S.A.). Then 15 ml scintillation fluid (Dimilume: Packard) were added 5 h later, and radioactivity in the embryos was determined in a liquid scintillation spectrometer (Packard 2000 CA) using luminescence and quench correction. Radioactivity was finally expressed as d.p.m./10 blastocysts (Niemann & Elsaesser, 1984). The background activity (20 μl of the last washing) was 40–55 d.p.m. per experiment and was subtracted from values obtained for embryos. The percentage of competitive inhibition was calculated as the difference between the total uptake of [^3H]oestrone and the uptake in the presence of a 100-fold excess of unlabelled oestrone, oestradiol-17 β , progesterone, testosterone or oestrone sulphate.

Experiment 2: retention of [^3H]oestrone and [^3H]progesterone. A total of 320 morulae was preincubated for 24 h (as described above), then cultured as blastocysts in groups of 10 for 6 h in the presence of 10 nM-[2,4,6,7- ^3H]oestrone or 10 nM-[1,2,6,7- ^3H]progesterone. After incubation, embryos were washed 3 times in KRB-solution. Accumulation of radioactivity by 10 groups was then determined, while further groups of blastocysts were cultured for another 30, 60 or 90 min in basic culture medium, before the net accumulation of radioactivity was established.

Experiment 3: oestrogen withdrawal from morulae. A total of 563 morulae was incubated in groups of 5–6, (1) in basic culture medium (control), (2) in medium supplemented with oestrone (1 nM or 100 nM), (3) in medium supplemented with nafoxidine (non-steroidal antioestrogen, 1,2-*p*-3,4-dihydro-methoxy-2-phenyl-1-naphthyl-phenoxythyl: Sigma No. 6632) at concentrations of 7.5 or 15 $\mu\text{g/ml}$, or (4) with 7.5 or 15 μg nafoxidine/ml plus different concentrations of oestrone (1 or 100 nM) or oestradiol-17 β (1 or 100 nM). Embryonic development was recorded after 24 h culture.

Nafoxidine was always dissolved in ethanol and prepared as a stock solution (1 $\mu\text{g}/\mu\text{l}$). The ethanol content of each medium was adjusted to 15.0 μl ethanol/ml.

Experiment 4: oestrogen withdrawal from blastocysts. A total of 272 blastocysts was incubated in groups of 5–6, (1) in basic culture medium (control), (2) in medium supplemented with 15 μg nafoxidine/ml, or (3) with 15 μg nafoxidine/ml plus 1 or 100 nM-oestrone or 1 or 100 nM-oestradiol-17 β . Blastocyst expansion was recorded after 24 h.

Statistical analysis. Differences between groups were calculated by one-way analysis of variance for completely randomized samples (Exps 1, 3 and 4) or by two-way analysis of variance for unbalanced data and the method of fitting constants (Exp. 2). Differences were considered to be significant at P values <0.05 . All data are expressed as mean $\pm$ s.e.m.

Results

Accumulation of [^3H]oestrone

After 6 h incubation, the uptake of [^3H]oestrone (or its metabolite(s)) was determined to be

550 ± 49 d.p.m. per 10 blastocysts (Fig. 1). Addition of a 100-fold excess of unlabelled oestrone or oestradiol-17 β to the culture medium reduced ($P < 0.001$) the uptake of radioactivity to 135 ± 14 d.p.m. or 148 ± 28 d.p.m., respectively, per 10 blastocysts. The percentage of competitive inhibition was therefore $74.6 \pm 1.5\%$ and $72.1 \pm 3.7\%$ for an excess of oestrone or oestradiol-17 β ,

Fig. 1. In-vitro accumulation (mean $\pm$ s.e.m.) of [3 H]oestrone by pig blastocysts in the absence or presence of a 100-fold excess of various steroids. The number of replicates, each involving 10 embryos, is indicated at the bottom of the bars.

Fig. 2. Percentage retention (mean $\pm$ s.e.m.) of radioactivity by pig blastocysts after pre-incubation with 10 nM-[3 H]oestrone or 10 nM-[3 H]progesterone. The number of replicates, each involving 10 embryos, is indicated at the bottom of the bars.

respectively. In contrast, the presence of a 100-fold excess of unlabelled progesterone, testosterone or oestrone sulphate did not affect the uptake of [^3H]oestrone ($P > 0.05$). Based on the specific activity of [^3H]oestrone (88.5 Ci/mmol) and an apparent specific uptake of 415 d.p.m. (550 minus 135 d.p.m.) per 10 blastocysts, the specific binding amounted to 2.1 fmol oestrone (or oestrone metabolite(s)) per 10 blastocysts.

Retention of [^3H]oestrone and [^3H]progesterone

At 30 min after termination of the incubation with [^3H]oestrone, $74.1 \pm 16.8\%$ of initial radioactivity was retained in the embryos, compared to $23.8 \pm 3.0\%$ retained radioactivity after incubation with [^3H]progesterone ($P < 0.001$) (Fig. 2). At 60 min of incubation, these values amounted to $66.0 \pm 10.4\%$ and $21.7 \pm 2.1\%$, respectively. Differences between levels of retained radioactivity at 30 and 60 min after incubation were not significant for either steroid. At 90 min after termination, levels of retained radioactivity were 55.3% and 13.5% respectively.

Fig. 3. Blastocyst formation rate (mean $\pm$ s.e.m.) during 24 h *in vitro* in the presence of 15 μg nafoxidine/ml plus oestrone or oestradiol-17 β . The number of replicates, each involving 5–6 embryos, is indicated at the bottom of the bars.

Oestrogen withdrawal from morulae

When morulae were cultured in the presence of 15 μg nafoxidine/ml, blastocyst formation was reduced ($P < 0.001$) to $21.5 \pm 8.9\%$ compared to controls ($98.3 \pm 1.7\%$) (Fig. 3). Nafoxidine used at a concentration of 7.5 $\mu\text{g}/\text{ml}$ did not affect blastocyst formation ($90.0 \pm 3.4\%$ development to blastocysts; data not shown in Fig. 3). The inhibitory effects of 15 μg nafoxidine/ml could be overcome in part by supplementation with 1 nm-oestradiol-17 β ($62.5 \pm 12.8\%$) and values were restored to $80.0 \pm 6.2\%$ when oestradiol-17 β was provided at a concentration of 100 nm. At both concentrations of supplementary oestradiol-17 β blastocyst formation was improved ($P < 0.001$) compared to the nafoxidine (15 $\mu\text{g}/\text{ml}$) group. Compared to controls, the blastocyst formation rate was lower ($P < 0.005$) after supplementation with nafoxidine plus 1 nm-oestradiol-17 β . In contrast, oestrone did not enhance blastocyst formation at 1 nm ($30.3 \pm 9.6\%$) or at 100 nm ($45.2 \pm 10.5\%$). Similarly, supplementary oestrone (1 nm or 100 nm) did not affect development of morulae to blastocysts in basic culture medium.

Fig. 4. Expansion (mean $\pm$ s.e.m.) after 24 h of pig blastocysts in the presence of 15 μ g nafoxidine/ml plus oestrone or oestradiol-17 β . The number of replicates, each involving 5–6 embryos, is indicated at the bottom of the bars.

Oestrogen withdrawal from blastocysts

Blastocyst expansion was decreased ($P < 0.01$) to $61.0 \pm 11.4\%$ in the presence of 15 μ g nafoxidine/ml compared to controls (100%) (Fig. 4). The rate of development amounted to $73.3 \pm 10.5\%$, $76.3 \pm 12.6\%$ or $85.5 \pm 5.2\%$ after addition of 100 nM-oestrone, or 1 nM- or 100 nM-oestradiol-17 β , respectively. The percentage of blastocyst expansion in culture medium supplemented with nafoxidine plus 100 nM-oestradiol-17 β was not different ($P > 0.05$) from control values.

Discussion

Aspects of the present results strongly suggest the presence of oestrogenic binding sites in the early pig embryo: for example, (1) the competitive inhibition of [3 H]oestrone (or its metabolite(s)) accumulation in the presence of a 100-fold excess of unlabelled oestrone or oestradiol-17 β ; (2) the lack of competitive effects in the presence of unlabelled progesterone, testosterone or oestrone sulphate; (3) the long-term retention of [3 H]oestrone; and (4) the inhibitory effects of the anti-oestrogen, nafoxidine, which could be reversed by oestradiol-17 β supplementation. Assuming that 1.2 μ g protein are present in a pig blastocyst (Wright *et al.*, 1983), the oestrogen binding calculated on a protein basis is approximately 175 fmol oestrone or oestrone metabolite(s) per mg protein. This is in the order of magnitude reported previously by us for the uptake of oestradiol-17 β by pig blastocysts (Niemann & Elsaesser, 1984) or the binding capacity of rat pituitary cytosol for oestradiol-17 β (van Beurden-Lamers *et al.*, 1974). Whether the apparent existence of an oestrogen binding protein in the blastocyst indicates binding of oestrogens as substrates to metabolizing enzymes or the presence of oestrogen receptors needs clarification.

Oestrogen receptors, or specific binding sites, have been suggested to be present in embryos from mice, rabbits and hamsters (Bhatt & Bullock, 1974; Holmes, 1976; Hutz *et al.*, 1984). In hamster embryos, uptake of oestradiol-17 β and progesterone was considerably reduced after super-ovulatory treatment which was explained by a possible saturation of receptors (Hutz *et al.*, 1984). Until now, we have no evidence that the steroid hormone uptake in the early pig embryo is affected

by a previous superovulatory treatment of the donor animals. Furthermore, the present results confirm the previous finding of a non-specific progesterone uptake (Niemann & Elsaesser, 1984) since, in contrast to oestrone, progesterone is rapidly released from the blastocysts.

Experiment 3 of this study identifies oestradiol-17 β as a potentially important factor in morula-blastocyst transformation. Although oestrone and oestradiol-17 β appear to be similar in their ability to compete with the uptake of [3 H]oestrone, oestrone was less effective than oestradiol-17 β in the restoration of the inhibitory effects of nafoxidine on morula-blastocyst transformation. With regard to the reduced biological activity of oestrone in this context, the affinity of oestrone for the presumptive oestrogen receptor may be less than that of oestradiol-17 β (Vreeburg *et al.*, 1975), or oestrone may not be active *per se* but acts through conversion to oestradiol-17 β , this conversion rate being low. At Days 14–18 of pregnancy, oestrone is the major unconjugated oestrogen formed from the radiolabelled precursors androstenedione, testosterone or DHA by the filamentous pig trophoblast, and oestrone is converted to other oestrogens including oestradiol-17 β (Gadsby *et al.*, 1980). However, at present it is not known whether zona pellucida-intact pig embryos are able to interconvert oestrone and oestradiol-17 β , nor is it known whether they synthesize oestrogens themselves. Day 1–3 mouse and rat embryos show activity of 17 β -hydroxysteroid dehydrogenase, the enzyme responsible for the interconversion of oestrone and oestradiol-17 β , and the catalysing direction was species-specific and dependent on the developmental stage of the embryo (Wu & Matsumoto, 1985). Furthermore, in 6-day-old rabbit blastocysts it was found that the enzyme mainly catalyses in the direction of oestrone to oestradiol-17 β conversion (Wu & Williams, 1986). Preliminary histochemical evidence for the presence of 17 β -hydroxysteroid dehydrogenase is also available for Day-5 pig blastocysts (Ishida & Nimura, 1977).

Testosterone, progesterone or oestrone sulphate did not retard accumulation of [3 H]oestrone (or its metabolite(s)) by pig blastocysts. Besides underlining the specificity of embryonic oestrogenic binding sites, this indicates that these steroids were not converted to active oestrogens in amounts able to compete with the accumulation of [3 H]oestrone. At later stages of pregnancy (Day 14), progesterone is converted to oestrone and oestradiol-17 β by pig blastocyst tissue and oestrone sulphate is rapidly hydrolysed mainly to oestrone (Perry *et al.*, 1973).

Whereas morula-blastocyst transformation is clearly dependent on oestrogens, the further development of pig blastocysts obviously is less. Roy *et al.* (1982) found that the addition of nafoxidine to the culture medium resulted in a complete inhibition of rat blastocyst expansion and trophoblast maturation which could be reversed by supplementation with oestradiol-17 β . We did not investigate trophoblast maturation of the pig blastocyst. It is, however, possible that oestrogens again become more effective in later blastocyst stages.

We thank Mr H.-H. Döpke for expert technical assistance. Parts of this study were supported by a grant from the Deutsche Forschungsgemeinschaft (DFG).

References

- Bhatt, B.M. & Bullock, D.W. (1974) Binding of oestradiol to rabbit blastocysts and its possible role in implantation. *J. Reprod. Fert.* **39**, 65–70.
- Gadsby, J.E., Heap, R.B. & Burton, R.D. (1980) Oestrogen production by blastocyst and early embryonic tissue of various species. *J. Reprod. Fert.* **60**, 409–417.
- Heap, R.B., Flint, A.P.F., Gadsby, J.E. & Rice, C. (1979) Hormones, the early embryo and the uterine environment. *J. Reprod. Fert.* **55**, 267–275.
- Holmes, P.V. (1976) Evidence for cytosol receptor protein specific for estradiol in the preimplantation blastocyst. *Proc. 8th Int. Congr. Anim. Reprod. & A.I., Cracow* **3**, 290–293.
- Hutz, R., Ghosh, M. & Dukelow, R. (1984) Steroid uptake and [3 H]uridine incorporation by early hamster and squirrel monkey (in vitro fertilized) embryos: effects of ovulatory regimen. *Zool. Sci.* **1**, 771–776.
- Ishida, K. & Nimura, S. (1977) Histochemical demonstration of steroid dehydrogenases in hamster and pig embryos at the preimplantation state. *Fert. Steril.* **28**, 350, Abstr.
- King, G.J. & Ackerley, C.A. (1985) Demonstration of oestrogens in developing pig trophectoderm and yolk sac endoderm between Days 10 and 16. *J. Reprod. Fert.* **73**, 361–367.
- Niemann, H. & Elsaesser, F. (1984) Uptake and effects of

- ovarian steroids in the early pig embryo: in vitro and in vivo studies. *Theriogenology* **21**, 84–102.
- Niemann, H. & Elsaesser, F. (1986) Evidence for estrogen-dependent blastocyst formation in the pig. *Biol. Reprod.* **35**, 10–16.
- Niemann, H., Illera, M.J. & Dziuk, P.J. (1983) Developmental capacity, size and number of nuclei in pig embryos cultured in vitro. *Anim. Reprod. Sci.* **5**, 311–321.
- Perry, J.S., Heap, R.B. & Amoroso, E.C. (1973) Steroid hormone production by pig blastocysts. *Nature, Lond.* **245**, 45–47.
- Roy, S.K., Sengupta, J., Paria, B.C. & Manchanda, S.K. (1982) In vitro inhibition of trophoblast maturation and expansion of early rat blastocysts by an oestrogen antagonist. *Acta endocr., Copenh.* **99**, 129–135.
- van Beurden-Lamers, W.M.O., Brinkmann, A.O., Mulder, E. & van der Molen, H.J. (1974) High affinity binding of oestradiol-17 β by cytosol from testis interstitial tissue, pituitary, adrenal, liver and accessory sex glands of the male rat. *Biochem. J.* **140**, 495–502.
- Vreeburg, J.T.M., Schretlen, P.J.M. & Baum, M.J. (1975) Specific, high-affinity binding of 17 β estradiol in cytosols from several brain regions and pituitary of intact and castrated adult male rats. *Endocrinology* **97**, 969–977.
- Wright, R.W., Jr, Grammer, J., Bondioli, K., Kuzan, F. & Menino, A. (1983) Protein content and volume of early porcine blastocysts. *Anim. Reprod. Sci.* **5**, 207–212.
- Wu, J.T. & Matsumoto, P.S. (1985) Changing 17 β -hydroxysteroid-dehydrogenase activity in preimplantation rat and mouse embryos. *Biol. Reprod.* **32**, 561–566.
- Wu, J.T. & Williams, K.J.H. (1986) Metabolism of estrogen by rabbit blastocysts in culture. *Biol. Reprod.* **34**, (Suppl.), 101, Abstr. 104.
- Zavy, M.T., Bazer, F.W., Thatcher, W.W., Wilcox, C.J. (1980) A study of prostaglandin F_{2 α} as the luteolysin in swine. V. Comparison of prostaglandin F, progestins, estrone and estradiol in uterine flushings from pregnant and nonpregnant gilts. *Prostaglandins* **20**, 837–851.

Received 1 September 1986