TIME AND NATURE OF THE SIGNAL FOR MATERNAL RECOGNITION OF PREGNANCY IN THE PIG

Promotor: dr. P.W.M. van Adrichem, hoogleraar in de algemene- en landbouwhuisdierfysiologie

Co-promotor: dr. F.A. Helmond, universitair hoofddocent, vakgroep Fysiologie van Mens en Dier

J. van der Meulen

TIME AND NATURE OF THE SIGNAL FOR MATERNAL RECOGNITION OF PREGNANCY IN THE PIG

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op dinsdag 19 december 1989
des namiddags te vier uur in de aula
van de Landbouwuinversiteit te Wageningen.

Stellingen

1. Voor het handhaven van de corpus luteum functie tijdens de dracht bij het varken, zijn tenminste twee opeenvolgende signalen vanuit de embryo's noodzakelijk.
   R.D. Geisert *et al.* (1988) *J. Reprod. Fert.* **79**, 163-172.
   Dit proefschrift.

2. Het ontbreken van het tweede signaal voor het handhaven van de corpus luteum functie tijdens de dracht, resulteert in het vertonen van berigheidsverschijnselen 25 tot 30 dagen na inseminatie.
   Dit proefschrift.

3. Het eerste signaal dat de embryo's van het varken genereren teneinde de corpus luteum functie te verlengen, moet meer omvatten dan alleen de afgifte van oestrogenen.
   Dit proefschrift.

4. Vanuit het gezichtspunt van de embryo's beschouwd is Maternal Recognition of Pregnancy niets anders dan een vorm van Material Requirements Planning.

5. Bij het paard is een extensieve mobiliteit van het embryo een vereiste voor het onderdrukken van endometrium-secretie van prostaglandine $F_{2\alpha}$.
   D.C. Sharp *et al.* (1989) *J. Reprod. Fert., Suppl.* **37**, 101-107.

6. Een initiële maternale reactie op bevruchting bij de muis is een reductie in het aantal trombocyten.
   C. O'Neill (1985) *J. Reprod. Fert.* **73**, 559-566.

7. Transcervicale uterus catheterisatie biedt de mogelijkheid om op niet-chirurgische wijze embryo's te verzamelen en te transplanteren.
   W. Hazeleger *et al.* (1989) *Theriogenology* (in press).

8. Indien men zou beseffen dat de lak van de auto ook te lijden heeft onder de zure regen, zouden alle 'heilige koeien' reeds lang met een katalysator uitgerust zijn.

9. Als we op het punt zijn gekomen dat een goede stervensbegeleiding voor moeder aarde het hoogst haalbare blijkt te zijn, kan het euthanasievraagstuk voorgoed in de ijskast worden gezet.
   De Volkskrant

10. Carpooling functioneert bij de gratie van de te overbruggen afstand.

11. In het kader van nieuwe milieuwetgeving zullen veevoeders NPK gekeurd moeten worden.

12. Een Optimist houdt altijd de wind in de zeilen.

J. van der Meulen

Time and nature of the signal for maternal recognition of pregnancy in the pig.

Wageningen, 19 december 1989

Aan mijn ouders
Aan Carin en Marc

Voorwoord

Het in dit proefschrift beschreven onderzoek is uitgevoerd binnen het kader van de Werkgroep "Vroege Dracht" bij de Vakgroep Dierfysiologie (vanaf februari 1989 geheten Vakgroep Fysiologie van Mens en Dier) van de Landbouwuniversiteit Wageningen. Graag wil ik iedereen bedanken die heeft bijgedragen aan het onderzoek en het tot stand komen van dit proefschrift.

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De discussies en samenwerking binnen de Werkgroep "Vroege Dracht" met Tette van der Lende, Henri Stroband, Truus te Kronnie, John Mattheij, Marleen Boerjan en Wouter Hazeleger hebben een belangrijke bijdrage geleverd aan het onderzoek. Tette van der Lende en Henry Stroband voorzagen het manuscript bovendien kritisch van commentaar.

De uitvoering van het onderzoek was niet mogelijk geweest zonder de inbreng van Corrie Oudenaarden, met name voor wat betreft de vele hormoon bepalingen. Op de "De Haar Varkens" was de verzorging, bronstcontrole en transport van de proefdieren was in vertrouwde handen bij Jan Hagens, Ries Verkerk, Andre Janssen, Sjaak Tijnagel en Ben van de Top. Op de Vakgroep Dierfysiologie werden de proefdieren verzorgd door Gijs van Gelderen, Dirk Vink, Martin Los en Anneke Fleurke. Zij verleenden ook assistentie bij de operaties en schonken vele malen een lekker "gezellig" bakje koffie. Willem Hofs regelde vele kleine boodschappen en op de technische vaardigheden van Tonny Roos en Leen Leenders werd nooit tevergeefs een beroep gedaan. Thea van Bemmel stond garant voor het typewerk, Joop van Brakel voor het verzorgen van tekenwerk en Anneke Helmond-Agema voor de correctie van de engelse tekst.

Een groot deel van het onderzoek is uitgevoerd in

samenwerking met doctoraalstudenten en stagiaires. Hun inzet en gezelligheid, ook buiten de normale werkuren, was enorm en werkte zeer stimulerend.

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Opgemerkt moet worden dat het onmogelijk is om in dit voorwoord iedereen te noemen die een bijdrage heeft geleverd, te meer omdat de bijdrage van enkelen niet met woorden te omschrijven is. Voor al degenen die niet persoonlijk genoemd zijn, hartelijk bedankt.

Contents

Chapter I

Introduction

Embryonic mortality

In the pig embryonic mortality, characterized by the number of missing or necrotic embryos relative to the number of corpora lutea (CL), is on average 20-40% and determines to a large extent total prenatal mortality (Flint *et al.*, 1982; Pope & First, 1985). The embryonic mortality rate may be influenced by the boar, the length of the period between parturition and insemination, the time of insemination relative to the time of ovulation, the feeding level and the occurrence of stress, but embryonic genetic abberations hardly account for the mortality (Van der Lende, 1989).

The major part of the embryos dies between Days 7 and 25 (Flint *et al.*, 1982; Pope & First, 1985). This may not be surprising in view of the complexity of physiological processes occurring during this stage of pregnancy: (a) the migration and spacing of the embryos in the uterus, (b) apposition and adhesion of the trophoblast with the uterine epithelium followed by attachment to the uterus (the process of 'implantation'), (c) increased capillary permeability and blood flow in the uterine vascular bed, and (d) prolongation of ovarian luteal function for maintenance of progesterone secretion (maternal recognition of pregnancy)(Dey *et al.*, 1984). This study has been focussed on the latter aspect.

Maternal recognition of pregnancy

With respect to the maternal recognition of pregnancy Short (1969) noted that 'one of the first outward and visible signs that an embryo has made its presence felt in the uterus is when the CL of the cycle becomes transformed into a CL of pregnancy, and oestrous cycles cease to recur'. The embryonic signal for the maternal recognition of pregnancy is certainly not the first embryonic sign, since e.g. a zygote induced protein complex has been detected in plasma of pregnant pigs as early as 24 h after insemination (Morton *et al.*, 1983).

Nevertheless, maintenance of pregnancy depends on the secretion of progesterone by the CL beyond the time when CL regression occurs during the oestrous cycle (Flint *et al.*, 1983). Progesterone is necessary for the secretion of endometrial histotrophe, which is essential to conceptus development. In the pig the CL remain the major source for progesterone for the whole duration of pregnancy (du Mesnil du Buisson & Dauzier, 1957).

During the oestrous cycle the life-span of the CL is limited by the action of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released by the uterine endometrium. Hysterectomy during the luteal phase of the oestrous cycle (du Mesnil du Buisson & Dauzier, 1959; Spies *et al.*, 1960; Anderson *et al.*, 1961) and destruction or absence of the uterine endometrium (Anderson *et al.*, 1969) result in CL maintenance. In cyclic gilts utero-ovarian vein (UOV) $PGF_{2\alpha}$ concentration is increased during the period of expected CL regression (Gleeson *et al.*, 1974; Killian *et al.*, 1976; Moeljono *et al.*, 1977; Frank *et al.*, 1977) and intramuscular (i.m.) injection of $PGF_{2\alpha}$ in hysterectomized (Moeljono *et al.*, 1976) and pregnant (Diehl & Day, 1974) gilts causes regression of the CL and cessation of progesterone secretion. In cyclic gilts CL function can be extended by administration of indomethacin, an inhibitor of prostaglandin synthesis (Kraeling *et al.*, 1981; Akinlosotu *et al.*, 1988).

During pregnancy, the luteolytic effect of $PGF_{2\alpha}$ is suppressed and prolongation of the CL life-span is achieved by an action of the developing embryos.

Early embryonic development

In the pig the embryos move from the oviducts into the uterine horns 2-3 days after fertilization (Perry & Rowlands, 1962; Oxenreider & Day, 1965). The embryos reach the blastocyst stage by Day 5, hatch from the zona pellucida on Day 6 (Perry & Rowlands, 1962) and distribute themselves over both horns from Day 7 (Dziuk, 1985). The diameter of the

spherical blastocysts increases from 0.5-1 mm at hatching up to 10 mm on Day 11 (Anderson, 1978; Stroband *et al.*, 1984). Thereafter elongation starts as a result of cellular remodelling and hypertrophy (Geisert *et al.*, 1982b). On Day 12 the blastocysts may display a variety of morphological forms. They can still be spherical or at different stages of elongation: ovoid, tubular or filamentous (Anderson, 1978). By Day 14 all blastocysts have achieved a thread-like filamentous form and they are 0.5-1 mm in diameter and up to 1 m long. During or shortly after elongation the blastocysts start to attach to the endometrial epithelium and follow the foldings of the endometrial surface, each occupying a relatively short part of the uterine horns (Perry & Rowlands, 1962). During the early elongation phase the blastocysts start to synthesize appreciable amounts of prostaglandins, proteins and oestrogens, which may be involved in the maternal recognition of pregnancy.

Blastocyst secretory activity

Prostaglandins. On Days 11-12 the prostaglandin content of blastocysts increases (Davis *et al.*, 1983). It is unlikely that this increase in blastocyst prostaglandin content is only caused by an increased capability to sequester prostaglandins from the uterine fluid, since it has been demonstrated that both younger (Stone *et al.*, 1986) and older blastocysts (Watson & Patek, 1979; Lewis *et al.*, 1983) have the capacity to synthesize prostaglandins. The major prostaglandin present in the blastocysts is prostaglandin E_2 (PGE_2)(Davis *et al.*, 1983; Stone *et al.*, 1986). Prostaglandins exert a wide array of effects on cellular and endocrine processes, including an increase in uterine bloodflow (Bazer & Roberts, 1983). Conflicting data exist concerning a possible antiluteolytic action of PGE_2 in the pig. Schneider *et al.* (1983) report no change in interoestrous interval of cyclic gilts by intrauterine PGE_2 injection. Intrauterine PGE_2 injections

according to a slightly different scheme, however, result in an extension of CL function (Akinlosotu *et al.*, 1986) and counteract the luteolytic effect of intrauterine injected $PGF_{2\alpha}$ in indomethacin treated gilts (Akinlosotu *et al.*, 1988).

Proteins. Between Days 10.5-18 the blastocysts secrete two major classes of proteins. Low molecular weight acidic proteins dominate between Days 10.5-12 and are secreted until Days 16-18. The major protein produced between Days 13-16, however, is a basic protein (Godkin *et al.*, 1982; Powell-Jones *et al.*, 1984; Baumbach *et al.*, 1988). The function of these proteins has not been established. An anti-luteolytic action of proteins as was observed in the ewe and cow (Bazer *et al.*, 1986), has not been detected in the pig (Harney, 1988). The secretory proteins do also not possess chorionic gonadotrophin-like activity (Powell-Jones *et al.*, 1984), although Saunders *et al.* (1980) demonstrated the presence of a chorionic gonadotrophin-like substance in extracts of embryos. It has been suggested that the secretory proteins may possess immunosuppressive, immunoneutralizing (Bazer & Roberts, 1983) and antiviral properties (Cross & Roberts, 1988).

Oestrogens. Conversion of labelled precursors into both oestrone and oestradiol-17ß during *in vitro* culture has demonstrated the ability of blastocysts to synthesize oestrogens (Perry *et al.*, 1973, 1976; Flint *et al.*, 1979; Gadsby *et al.*, 1980). This aromatase activity is initiated in large spherical Day-11 blastocysts (Fischer *et al.*, 1985; Mondschein *et al.*, 1985). In uterine flushings total recoverable oestrogen increases until Day 12 and decreases by Day 14 (Zavy *et al.*, 1980; Geisert *et al.*, 1982a); in the blastocysts oestrogen concentrations show a similar pattern (Flint *et al.*, 1983). The release of oestrogens by the blastocysts stimulates the synchronous release of secretory vesicles (histotrophe) from the endometrial epithelium into the uterine lumen (Geisert *et al.*, 1982a, 1982c; Young *et al.*, 1987). In this way a complex *in vivo* 'culture medium' is established to promote conceptus development. Oestrogens not only induce the release of histotrophe, but also seem to

lengthen the period of histotrophe production by exerting an anti-luteolytic effect to continue progesterone secretion.

Anti-luteolytic action of oestrogen

I.m. injection of large doses of diethylstilbestrol (Kidder *et al.*, 1955), oestradiol valerate (Frank *et al.*, 1977, 1978) or oestradiol benzoate (Ziecik *et al.*, 1986) in cyclic gilts once on Day 11 or daily on Days 11-15, results in prolongation of CL function for a variable time period. In such gilts, plasma progesterone level (Bazer *et al.*, 1982) and uterine endometrial function (Basha *et al.*, 1980a, 1980b; Geisert *et al.*, 1982c) did not seem to be different from that of pregnant gilts, and UOV plasma $PGF_{2\alpha}$ concentration was lower compared with cyclic gilts (Frank *et al.*, 1977). Several mechanisms have been proposed to explain this anti-luteolytic action of oestrogens in the pig.

Bazer & Thatcher (1977) suggest an oestrogen induced alteration in the direction of movement of $PGF_{2\alpha}$ from the epithelial cells of the uterine endometrium. During the late luteal phase of the oestrous cycle $PGF_{2\alpha}$ is secreted in an endocrine direction, resulting in an increased UOV $PGF_{2\alpha}$ concentration (Gleeson *et al.*, 1974; Moeljono *et al.*, 1977; Frank *et al.*, 1977; Killian *et al.*, 1976). During pregnancy $PGF_{2\alpha}$ is secreted in an exocrine direction. It accumulates in the uterine lumen (Zavy *et al.*, 1980) and fails to enter the uterine vasculature (Moeljono *et al.*, 1977; Killian *et al.*, 1976).

Using a perifusion device, Gross *et al.* (1988) demonstrated that endometrium from cyclic gilts secretes $PGF_{2\alpha}$ primarily from the myometrial side and that pregnant gilts secrete $PGF_{2\alpha}$ from the luminal side. The switch in direction of $PGF_{2\alpha}$ secretion from an endocrine to an exocrine orientation is closely associated with a period of release and re-uptake of calcium by the endometrium in pregnant gilts, which is initiated by exogenous oestrogen as well as endogenous

oestrogen. Treatment of endometrium from Day-14 cyclic gilts in the perifusion device with a calcium ionophore (an inducer of calcium cycling by epithelium) switches secretion of $PGF_{2\alpha}$ from an endocrine to an exocrine mode, and suggests that induction of calcium cycling across endometrial epithelium is responsible for the redirection of $PGF_{2\alpha}$ secretion toward the uterine lumen (T.S. Gross & F.W. Bazer, unpublished data, quoted by Bazer *et al.*, 1989).

Ford & Christenson (1979) suggest a dilution of secreted $PGF_{2\alpha}$ by an oestrogen induced increase in uterine blood flow (UBF). During pregnancy UBF is similar to that during the oestrous cycle, until Days 12-13 when a 3- to 4-fold increase in flow occurs (Ford & Christenson, 1979). Increase in UBF is a consequence of a catechol oestrogen mediated decrease in Ca^{2+} uptake by uterine arterial smooth muscle cells, resulting in a decreased uterine arterial tone (Stice *et al.*, 1987a, 1987b). From Days 12-13 blastocysts not only synthesize oestrogens, but also develop the ability to convert oestrogens into catechol oestrogens (Mondschein *et al.*, 1985). The catechol oestrogens reach the uterine artery via a lymphatic route (Magness & Ford, 1982, 1983).

A change in back transfer of $PGF_{2\alpha}$ into the uterine horns has been suggested by Krzymowski *et al.* (Koziorowski *et al.*, 1986; Krzymowski *et al.*, 1986). $[^3H]PGF_{2\alpha}$, infused into the myometrium or injected into the uterine lumen, flows out from the uterus with the venous blood and lymph. It penetrates from the vein and lymphatic vessels into the arterial blood in the area of the mesometrium, and is transferred back into the uterine horns (Koziorowski *et al.*, 1986; Krzymowski *et al.*, 1986, 1987). This back transfer is strongly reduced on Days 13-17 of the oestrous cycle (Krzymowski *et al.*, 1986). A high level of oestradiol, generated endogenously by the blastocysts or i.m injected on Days 11-15, results in a dilation of the uterine arterial branches in the ligamentum latum and prompts an increase of $PGF_{2\alpha}$ transfer into the arterial blood (Krzymowski *et al.*, 1986, 1987). Moreover, oestradiol may increase binding of $[^3H]PGF_{2\alpha}$ in the ligamentum latum and the

uterus (Krzymowski *et al.*, 1987).

An alternative mechanism by which large doses of injected oestrogen may act anti-luteolytic, involves an effect on the CL exerted either directly or via an extra-uterine organ (Flint *et al.*, 1983). Oestradiol administration results in both intact and hysterectomized gilts in an increase in LH receptor level in the CL (Garverick *et al.*, 1982). This effect may be mediated by prolactin, which increases after oestradiol treatment (Ziecik *et al.*, 1986) and which synergizes with chorionic gonadotrophin to increase progesterone production by luteal cells *in vitro* (Flint *et al.*, 1983).

Aim of this study

The object of this study was (a) to determine the time of the maternal recognition of pregnancy in the pig, (b) to measure oestrogen release by the blastocysts during this time period and (c) to investigate the effect of locally administered physiological doses oestradiol-17ß on CL function in cyclic gilts.

The time period at which the maternal recognition of pregnancy takes place has been determined by comparing progesterone profiles of cyclic and pregnant gilts (chapter III) and by removing blastocysts from both horns of gilts on different days of pregnancy (chapter IV). In order to reduce possible differences in the time of ovulation and CL development between gilts, injection of a GnRH analogue at the time of observing first standing oestrus (Van der Meulen *et al.*, 1986; chapter II) has been used, in the experiments described in chapter III and IV.

The participation of each blastocyst at the initiation of oestrogen production has been determined by measurement of aromatase activity in individual spherical blastocysts (chapter V). The oestrogen synthesis capacity of blastocysts during the time of the maternal recognition of pregnancy has been determined by measuring oestrogen release during *in vitro*

cultures of spherical and filamentous blastocysts (chapter VI).

Endometrial oestrogen receptor concentrations have been determined around the time of expected maternal recognition of pregnancy in cyclic and pregnant gilts (chapter VII). The effect of locally administered physiological doses of oestrogen on CL function has been determined by intrauterine oestradiol-17ß injection and by intrauterine insertion of oestradiol-17ß releasing micropellets in cyclic gilts (chapter VIII).

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CHAPTER II

Effect of injection of a GnRH analogue at the onset of oestrus on LH and FSH release and embryonic mortality in gilts

J. van der Meulen[1], T. van der Lende[2], G. te Kronnie[3] and F.A. Helmond[1]

[1]Department of Animal Physiology, Agricultural University, Haarweg 10, 6709 PJ Wageningen, The Netherlands;
[2]Department of Animal Husbandry, Agricultural University, Marijkeweg 40, 6709 PG Wageningen, The Netherlands;
[3]Department of Experimental Animal Morphology and Cell Biology, Agricultural University, Marijkeweg 40, 6709 PG Wageningen, The Netherlands

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Summary

The effect of i.m. injection of 100 μg GnRH analogue at the onset of oestrus on LH and FSH release and number of corpora lutea (CL) and embryos in gilts was investigated. In *experiment I*, the LH and FSH peak of 5 GnRH treated gilts were not significantly different compared with the previous control oestrous period. Both LH and FSH peak, however, tended to be sharper after GnRH injection. In *experiment II*, GnRH treated and control gilts were slaughtered on Days 4, 11, 13 or 35 of pregnancy. In 15 GnRH treated gilts slaughtered on Days 4, 11 or 13 the number of CL (16.3±2.3; mean ± s.d.) and embryos (15.0±3.0) were significantly increased ($P<0.05$) compared with non-treated control gilts (N=15; CL: 14.8±1.5; embryos: 12.1±3.4). The embryonic mortality rate in the Day-35 control group was relatively low (11.3%) compared with the embryonic mortality rate in the Day 4, 11 and 13 control group. The embryonic mortality in the GnRH group was increased to 24.7% on Day 35. Including the data of the gilts slaughtered on Day 35, the number of CL and embryos in the GnRH group were larger than in the control group (CL: 16.4±2.3 and 15.4±2.0, respectively; embryos 14.3±3.3 and 13.0±3.3, respectively) but this was no longer significant. The results of this study indicate that injection of a GnRH analogue at the time of observing first standing oestrus in cyclic gilts induces a sharper LH and FSH peak and tends to increases the number of ovulations and embryos.

Introduction

Single or repeated GnRH injections can induce an LH peak and subsequent ovulation in prepuberal gilts (Chakraborty *et al.*, 1973; Lutz *et al.*, 1985), gilts with a delayed puberty (Edqvist *et al.*, 1978), lactating sows (Cox & Britt, 1982) and anoestrous sows (Armstrong & Britt, 1985). Administration of GnRH at the onset of oestrus may also affect ovulation rate

and/or embryonic survival. An i.v. injection of 200 µg GnRH at the onset of puberal oestrus in gilts, resulted in an increase in the number of corpora lutea (CL) but did not enhance the number of viable embryos on Day 30 of pregnancy (Archibong *et al.*, 1987). In high-fed sows an i.m. injection of 50 µg GnRH at the onset of first postweaning oestrus did not affect the number of CL or viable embryos, but in first-parity low-fed sows GnRH injection resulted in an increased number of viable embryos on Day 25 of pregnancy (Kirkwood *et al.*, 1987).

In this study a GnRH analogue was injected at the onset of oestrus in cyclic gilts. The effect of GnRH injection on preovulatory LH and FSH concentrations and on embryonic mortality and development on Days 4, 11, 13 and 35 of pregnancy was investigated.

Materials and methods

Experiment I. Five crossbred gilts (Great Yorkshire x Dutch Landrace) which had shown 3 normal oestrous cycles (18-22 days) were fitted with an indwelling jugular vein catheter (PVC, 1.0 mm i.d., 1.5 mm o.d.) 5 days before expected oestrus. Blood samples were collected 3 times daily (9:00, 12:00 and 15:00 h). The gilts were checked for oestrus with a vasectomized boar twice daily (09:00 and 15:00 h). At the onset of standing oestrus (Day 0) 2 ml saline were injected i.m. (control oestrus). Blood samples were collected at 30 min intervals for the first 4 h following the injection and at 6, 9, 12 and 15 h thereafter. In the next cycle of these 5 gilts, at the time of observing first standing oestrus, 100 µg GnRH analogue (Ovalyse: Upjohn Company, Ede, The Netherlands) were injected i.m. (GnRH oestrus). Blood samples were taken in the same frequency as in the previous (control) oestrous period.

Experiment II. Forty-nine crossbred gilts (Great Yorkshire x Dutch Landrace) were checked for oestrus with a vasectomized boar twice daily (09:00 and 15:00 h). Before the second

oestrus the gilts were randomly assigned to one of 2 groups. In the GnRH group the gilts were injected i.m. with 100 µg GnRH analogue at the time of observing first standing oestrus (Day 0). In the control group the gilts were not treated. All gilts were artificially inseminated with fresh diluted semen on Day 1, 24 h after observing first standing oestrus.

Gilts from both groups were slaughtered on Days 4 (N=11), 11 (N=13), 13 (N=11) or 35 (N=14) of pregnancy. These days were chosen in order to evaluate mortality rate before blastocyst formation (Day 4), before elongation (Day 11), after elongation (Day 13) and at the end of the embryonic stage (Day 35). From each gilt the reproductive tract was removed and transported on ice to the laboratory. The number of CL on each ovary was determined, and the uterus and cervix were separated from the mesometrium.

On Day 4 the oviducts and uterine horns were flushed twice with 10 and 30 ml Dulbecco's phosphate-buffered saline (PBS: Gibco, Paisley, UK), respectively, and the number of embryos was determined.

On Day 11 the uterine horns were flushed and the number of blastocysts was determined and their diameter was measured.

On Day 13 the uterine horns were opened longitudinally along the antimesometrial border, placed in a dissection tray in Dulbecco's PBS and pinned 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). In this way filamentous blastocysts were recovered individually. The number of blastocysts was determined after they had been checked for the presence of an embryoblast.

On Day 35 the uterine horns were opened longitudinally and the embryos were removed from the uterus (Van der Lende, 1989). The number of apparently normal and healthy embryos was determined. The weight of the embryos and the extra-embryonic membranes and the length of the embryos and placentae was measured (Van der Lende, 1989).

Hormone analysis. All blood samples were collected into

heparinized tubes and centrifuged. The separated plasma was stored at -20°C until hormone analysis.

Plasma concentrations of LH were measured by a double-antibody RIA (Niswender *et al.*, 1970), using porcine LH (LER 786-3, potency 0.65 x NIH-LH-S1) as a standard and for iodination. Anti-ovine LH 614 IV was used at a 1:30000 working dilution and goat anti-rabbit immunoglobulin as the second antibody. The sensitivity of the assay was 0.7 ng/ml at the 90% B/BO level. The intra-assay coefficient of variation was 13.8 % and the inter-assay coefficient of variation was 16.8%.

Plasma FSH concentrations were measured in a similar way as plasma LH, using antiserum to porcine FSH (i531/001: UCB, Brussels, Belgium, working dilution 1:15000) and porcine FSH (i031: UCB, potency 280 x NIH-FSH-P1) as a standard and for iodination. The sensitivity of the assay was 1.9 ng/ml at the 90% B/BO level. The intra-assay coefficient of variation was 8.2% and the inter-assay coefficient of variation was 11.0%.

Plasma concentrations of progesterone were measured by RIA (Helmond *et al.*, 1980), using a specific rabbit antiserum against 4-pregnene-6ß-ol-3,20-dione-hemisuccinate-BSA. The main cross-reacting steroids were pregnenolone (98.0%), corticosterone (2.7%), 17α-hydroxyprogesterone (1.5%) and 20α-hydroxy-4-pregnen-3-one, cortisol, oestrone, oestradiol-17α, oestradiol-17ß, oestriol, androstenedione, testosterone and dehydroandrosterone (all <0.2%). The antiserum was used in a working dilution of 1:15000. The intra-assay coefficient of variation was 9.6% and the inter-assay coefficient of variation was 9.8%.

Statistical analysis. Hormone analysis data were transformed to their natural logarithms and differences in LH, FSH and progesterone between the control and GnRH oestrous period were tested for significance by Student's paired *t* Test. Differences in number of CL, embryos and embryonic mortality rate between the control and GnRH group were tested for significance by analysis of variance (SAS Institute Inc., 1985). All results are expressed as mean ± s.d.

Results

In *experiment I*, during both oestrous periods standing oestrus was first detected at the 9:00 h oestrous check. The LH concentration during the 2 pre-oestrous days was the same in control (1.8±0.6 ng/ml) and GnRH oestrus (1.9±0.6 ng/ml). At the time of observing first standing oestrus LH was increased in both control (4.5±3.1 ng/ml) and GnRH oestrus (4.1±1.8 ng/ml). In the control oestrus LH was at its maximum 1 h after observing first standing oestrus (4.9±3.0 ng/ml) and remained around 4.4 ng/ml for another 3 h. In the GnRH oestrus LH showed a further increase up to 2 h after GnRH injection (6.6±3.0 ng/ml) and declined thereafter (Fig. 1).
The FSH concentration during the 2 pre-oestrous days in the control oestrus (9.0±4.4 ng/ml) was higher than in the GnRH oestrus (5.0±3.7 ng/ml)($P<0.01$). In the control oestrus FSH was increased at the time of observing first standing oestrus (14.7±4.6 ng/ml) and remained around this level with a maximum value (18.9±6.5 ng/ml) 3 h after injection of saline. In the GnRH oestrus FSH was also increased at the time of observing first standing oestrus (10.5±7.3 ng/ml), increased to 17.0±8.7 ng/ml 2.5 h after GnRH injection, and declined thereafter. The FSH peak occurred 30 minutes after the LH peak. A second FSH peak occurred on Day 3 in the control oestrus (24.9±7.5 ng/ml) and on Day 2 in the GnRH oestrus (22.2±5.2 ng/ml) (Fig. 1).
During both oestrous periods progesterone increased to levels above 1 ng/ml on Day 2 (Fig. 1).

At slaughter on Days 4, 11 and 13 in *experiment II*, one control and 4 GnRH treated gilts appeared to be non-pregnant and 2 gilts of the Day-35 control group returned to oestrus before slaughter. The pregnancy rate in the control group was 87.5% and in the GnRH group 84.0%. The data concerning the CL numbers of the non-pregnant gilts were excluded from analysis.
On Day 4 the number of CL in the GnRH group was significantly larger than in the control group ($P<0.05$), but there was no significant difference for the number of embryos (Table 1).
On Days 11 and 13 the number of CL and embryos was larger in

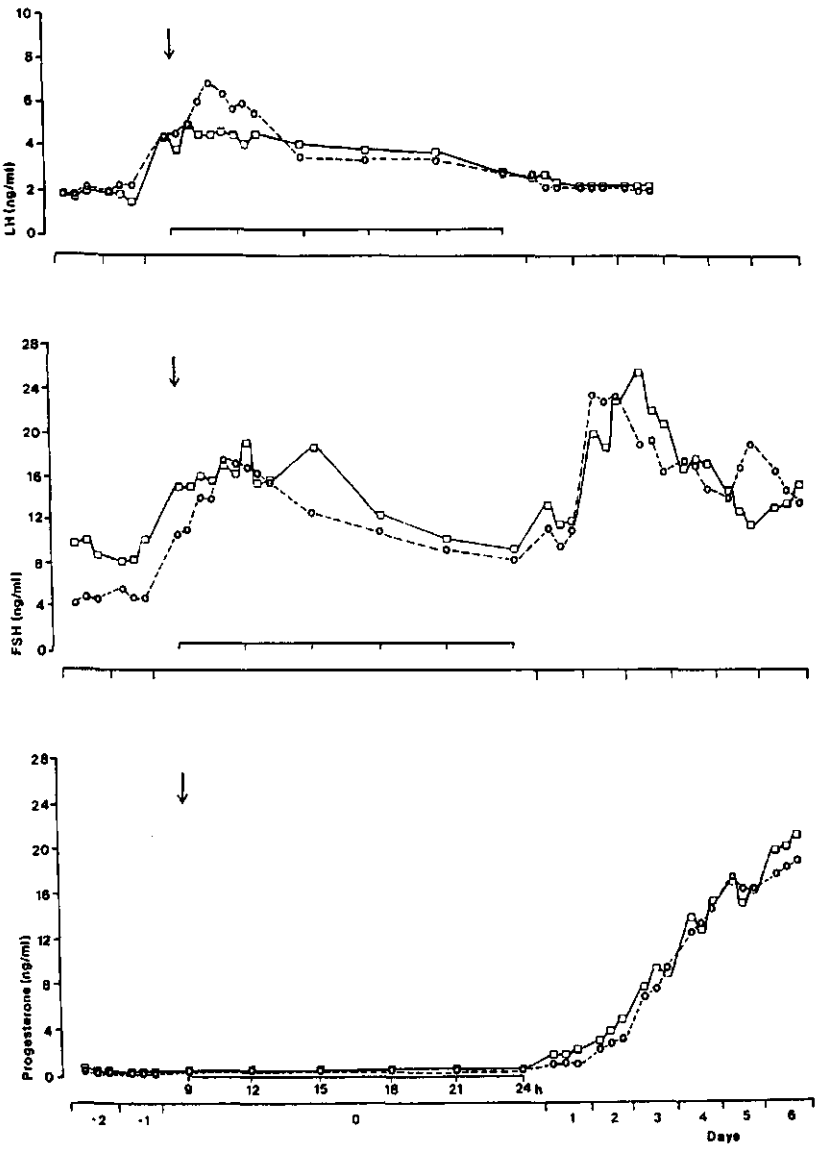

Fig. 1. Plasma concentrations of LH, FSH and progesterone in 5 gilts during 2 successive oestrous periods. Saline (□-□) was injected in the first period at the onset of standing oestrus (Day 0,↓) and a GnRH analogue (O-O) was injected in the second period.

Table 1. The number of CL and embryos and the embryonic mortality rate of control and GnRH treated gilts slaughtered on Days 4, 11, 13 and 35 of pregnancy.

Group	Day of slaughter	N	Number of CL	Number of embryos	Mortality rate (%)
Control	4	5	15.0±0.7[a]	13.0±2.0	13.5±11.2
GnRH	4	5	17.2±1.9[b]	15.2±3.3	12.3±10.3
Control	11	5	14.6±2.3	12.4±3.8	16.6±18.3
GnRH	11	5	15.0±1.6	13.6±1.8	9.2± 8.9
Control	13	5	14.8±1.3	11.0±4.4	25.8±29.8
GnRH	13	5	16.8±3.0	16.2±3.6	5.8± 1.4
Control	35	6	17.0±2.5	15.2±2.1	11.3± 9.7
GnRH	35	6	16.7±2.5	12.5±3.5	24.7±20.5

Values with different superscripts are different ($P<0.05$)

the GnRH group than in the control group, but these differences were not significant (Table 1). On Day 35 the opposite was observed; a not significantly larger number of CL and embryos in the control group compared with the GnRH group (Table 1).

For all the gilts slaughtered on Days 4, 11 and 13 the number of CL and embryos in the GnRH group was larger than in the control group ($P<0.05$) and the embryonic mortality rate was lower (Table 2). Including the data of gilts slaughtered on Day 35, the number of CL and the number of embryos were still larger in the GnRH group than in the control group, but this was no longer significant (Table 2).

The cell number of Day-4 embryos varied between 1 and 9. The number of 1-cell embryos did not significantly differ between

Table 2. The number of CL and embryos and the embryonic mortality rate in the control and GnRH group.

	N	Number of CL	Number of embryos	Mortality rate (%)
		Days 4, 11 and 13		
Control	15	14.8±1.5[a]	12.1±3.4[a]	18.6±20.4
GnRH	15	16.3±2.3[b]	15.0±3.0[b]	9.1± 8.9
		Days 4, 11, 13 and 35		
Control	21	15.4±2.0	13.0±3.3	16.5±18.0
GnRH	21	16.4±2.3	14.3±3.3	13.6±14.6

Values with different superscripts are different ($P<0.05$)

both groups (control group: 4 1-cell embryos in 3 gilts; GnRH group: 7 1-cell embryos in 3 gilts).

On Day 11 the recovered blastocysts were all spherical with a diameter of 4.8±1.1 (control group) and 4.6±0.6 mm (GnRH group). The variation in blastocyst diameter within litters was larger in the control group than in the GnRH group (1.1±0.5 versus 0.7±0.1 mm). However, after correction for differences in blastocyst diameter this difference in within-litter variation was not significant ($P=0.068$).

On Day 13 most of the recovered blastocysts were filamentous. In the control group in 4 gilts 1 or 2 blastocysts were tubular. In the GnRH group in 2 gilts blastocysts were spherical, tubular and filamentous.

On Day 35 the weight of the embryos and extra-embryonic membranes and the length of the embryos and placentae were larger in the GnRH treated gilts than in the control gilts, but none of these differences was significant (Table 3).

Table 3. Parameters for embryonic development in the GnRH and control group on Day 35 of pregnancy.

	Control-group	GnRH-group
embryonic weight (g)	3.7±0.4	4.0±0.3
embryonic length (cm)	3.9±0.2	4.0±0.2
placental weight (g)	33.6±5.6	38.1±12.3
placental length (cm)	36.8±25	42.1±8.1
amniotic fluid weight (g)	4.6±0.4	4.9±0.5
allantoic fluid weight (g)	86±47	131±87

Discussion

The magnitude of the maximum LH level during control and GnRH oestrus, in *experiment I*, did not differ from those reported before (Henricks *et al.*, 1972; Wilfinger *et al.*, 1973a; Parvizi *et al.*, 1976; Brinkley, 1981; Van de Wiel *et al.*, 1981). In previous studies, a preovulatory FSH peak occurring almost simultaneously with the preovulatory LH peak was usually observed in gilts (Vandalem *et al.*, 1979; Van de Wiel *et al.*, 1981) and sows (Edwards and Foxcroft, 1983), although in some studies this FSH peak was not detected (Wilfinger *et al.*, 1973b; Rayford *et al.*, 1974; Brinkley, 1981).

The maximum LH and FSH levels did not differ between the control and GnRH oestrus. Compared with the control oestrus, however, both LH and FSH peaks tended to be sharper after injection of a GnRH analogue. Since in the control oestrus the LH peak in all 5 gilts occurred within 4 h after observing first standing oestrus, a sharper LH peak may be the result of endogenous LH release enhanced by GnRH. In anoestrous sows induced to ovulate by pulsatile GnRH administration, the induced LH peak also seems sharper compared with that of sows

exhibiting spontaneous oestrus (Armstrong & Britt, 1985).

In *experiment II*, the number of CL in GnRH treated gilts was on average 1 larger than in control gilts. In gilts injected with GnRH at the onset of puberal oestrus (Archibong *et al.*, 1987) and in GnRH treated first-parity low-fed sows (Kirkwood *et al.*, 1987) the number of CL was increased by about 2.5. This increase in number of CL is due to the ovulation of follicles which would not have ovulated without GnRH injection, and which in turn may be related to the sharper LH and FSH peak.

On Days 4, 11 and 13 the higher ovulation rate in the GnRH group was accompanied by a decreased embryonic mortality rate and an increased number of embryos. The possible occurrence of a delayed LH peak with respect to the onset of oestrus (Tilton *et al.*, 1982; Ziecik *et al.*, 1982; Helmond *et al.*, 1986) may have been prevented by the injection of the GnRH analogue. In this way potential mortality due to a lack of synchrony between the time of the preovulatory LH peak and the onset of oestrus (Tilton *et al.*, 1982; Helmond *et al.*, 1986) may have been reduced. Since the variation in diameter of the Day-11 blastocysts was lower in GnRH treated gilts than in control gilts, the induced sharper preovulatory LH peak may also have affected the duration or pattern of ovulation and as a consequence embryonic survival (Pope *et al.*, 1988).

In this study, the number of blastocysts was not only determined before (Day 11) but also just after elongation (Day 13), in order to evaluate a possible effect of this process on mortality rate. The mortality rate in the GnRH group did not change from Day 11 to 13, and was comparable to the mortality rate on Day 4. This indicates that in the GnRH group the preimplantation embryonic mortality occurred before Day 4. In the control group embryonic mortality increased during the process of elongation.

On Day 35 the number of CL and embryos in the control group exceeded those of GnRH treated gilts. In the Day-35 control group, however, the number of CL was relatively high and the embryonic mortality rate of 11.3 % was low compared with the

embryonic mortality rate in the Day 4, 11 and 13 control groups, the embryonic mortality rate in other groups of the same breed on Day 35 at our laboratory and those described earlier for animals which were not treated with GnRH (Flint *et al.*, 1982; Pope & First, 1985; Van der Lende, 1989). On Day 35 the embryonic mortality rate in the GnRH group was 24.7%. This is increased compared with Days 4, 11 and 13, but comparable with mortality rates described earlier (Flint *et al.*, 1982; Pope & First, 1985; Van der Lende, 1989). The reason for this increase in embryonic mortality rate in the GnRH group after elongation (Day 13) and Day 35 is unknown. The difference in embryonic development on Day 35 between the control and GnRH group is small, and there were no indications that the variation within litters differed between these 2 groups. The difference in placental development reflects differences in embryonic mortality rate (Van der Lende, 1989).

The increased number of CL after GnRH injection without a concomitant increase in embryonic mortality rate, may result in an increase in the number of embryos in cyclic gilts treated with GnRH. Such an increase in number of embryos was also observed in low-fed first-parity sows injected with GnRH at the onset of oestrus (Kirkwood *et al.*, 1987), but not in gilts treated with GnRH at the onset of puberal oestrus (Archibong *et al.*, 1987). Therefore, it may be concluded that injection of a GnRH analogue at the time of observing first standing oestrus in cyclic gilts results in a sharper LH and FSH peak, and both the number of CL and embryos are slightly increased.

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Chapter III

Corpus luteum maintenance in the pig: progesterone profiles of cyclic and pregnant gilts, and case studies of 2 gilts in which pregnancy failed

J. van der Meulen, F.A. Helmond and C.P.J. Oudenaarden

Department of Animal Physiology, Agricultural University,
Haarweg 10, 6709 PJ Wageningen, The Netherlands

submitted for publication

Summary

Progesterone profiles of pregnant and cyclic gilts were compared. From Day 14 a significant difference in progesterone concentration existed between cyclic (N=7) and pregnant gilts (N=6)($P<0.05$). This indicates that corpus luteum (CL) rescue during pregnancy occurs before Day 14. In one of the inseminated gilts the progesterone concentration declined on Day 27 and the gilt returned to oestrus on Day 30. The progesterone and oestrone sulphate profiles of this gilt indicated that return to oestrus may have been caused by embryonic loss before Day 14 after a first signal for CL maintenance has been generated. Another gilt aborted on Day 27. The data of this gilt indicate that pregnancy failure may have been caused by a maternal malfunction.

Introduction

During pregnancy in the pig, progesterone is necessary for the secretion of endometrial histotrophe (required for blastocyst growth) and suppression of uterine contraction activity.

There is a minimum daily progesterone requirement for the maintenance of pregnancy of 4-5 ng/ml (Ellicott & Dziuk, 1973). A decrease of progesterone below this level by ovariectomy (Ellicott & Dziuk, 1973; First & Staigmiller, 1973; Nara *et al.*, 1981), removal of the corpora lutea (CL)(Nara *et al.*, 1981) or injection of $PGF_{2\alpha}$ (Diehl & Day, 1974) results in termination of pregnancy and return to oestrus. For the whole duration of pregnancy the CL are the main source of progesterone. At least 5 CL are needed for the production of sufficient progesterone to maintain pregnancy (Du Mesnil du Buisson & Dauzier, 1959; Martin *et al.*, 1977).

A prerequisite for the establishment and maintenance of pregnancy is the rescue of the CL in early pregnancy. In non-pregnant pigs the life-span of the CL is limited by the

luteolytic action of $PGF_{2\alpha}$ (Bazer *et al.*, 1982), which is elevated in the utero ovarian vein (UOV) between Days 13-17 (Moeljono *et al.*, 1977). As a result of an anti-luteolytic action of the blastocysts UOV $PGF_{2\alpha}$ concentration remains low during pregnancy and $PGF_{2\alpha}$ is prevented to reach the CL in sufficient amounts to cause luteal regression. In this way progesterone secretion continues.

In this study the time of maintenance of CL function was investigated by measuring progesterone concentrations of cyclic and pregnant gilts. The progesterone and oestrone sulphate concentrations of 2 gilts in which pregnancy failed before Day 30 were compared with those of pregnant gilts.

Materials and Methods

Animals and experimental design. Fifteen crossbred gilts (Great Yorkshire x Dutch Landrace) which had shown 2 or more normal oestrous cycles (18-22 days) were used. The gilts were checked for oestrus with a vasectomized boar twice daily (09:00 and 15:00 h). At least 5 days before expected oestrus, they were fitted with an indwelling jugular vein catheter (PVC, 1.0 mm i.d., 1.5 mm o.d.). Blood samples were collected around oestrus three times a day (09:00, 12:00 and 15:00 h) and from Day 4 once each day (09:00 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. Artificial insemination (AI) was carried out in 8 gilts on Day 1, about 26 h after observing first standing oestrus (Van der Meulen *et al.*, 1986). Blood sampling and checking for oestrus continued, until the cyclic gilts (N=7) returned to oestrus and until the pregnant gilts (N=8) were slaughtered on Day 35.

One pregnant gilt (No. 161) returned to oestrus on Day 30 and another pregnant gilt (No. 11) aborted on Day 28.

Hormone analysis. All blood samples were collected into

heparinized tubes and centrifuged; plasma was stored at -20°C until further analysis.

Plasma concentrations of progesterone were measured in all samples by RIA (Helmond *et al.*, 1980), using a specific rabbit antiserum against 4-pregnene-6ß-ol-3,20-dione-hemisuccinate-BSA. The main cross-reacting steroids were pregnenolone (98.0%), corticosterone (2.7%), 17α- hydroxyprogesterone (1.5%) and 20α-hydroxy-4-pregnen-3-one, cortisol, oestrone, oestradiol-17ß, oestradiol-17α, oestriol, androstenedione, dehydroandrosterone and testosterone (all <0.2%). The antiserum was used in a working dilution of 1:15000. The sensitivity of the assay was 0.1 ng/ml at the 90% B/B_0 level. The intra-assay coefficient of variation was 7.1% and the inter-assay coefficient of variation was 12.5%.

Plasma concentrations of LH were measured in the samples collected around oestrus by a double-antibody RIA (Niswender *et al.*, 1970), using pig LH (LER 786-3, potency 0.65 x NIH-LH-S1) as a standard and for iodination. Anti-ovine LH 614 IV was used at a 1:30000 working dilution and goat anti-rabbit immunoglobulin as the second antibody. The sensitivity of the assay was 0.7 ng/ml at the 90% B/B_0 level. The intra-assay coefficient of variation was 13.8% and the inter-assay coefficient of variation was 15.1%.

Plasma concentrations of oestrone sulphate were measured in all samples by RIA after extraction and column chromatography, using a modification of the method described by Helmond *et al.* (1980). Samples of 100 µl 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). The samples were incubated overnight at 37°C in the dark after addition of an enzyme preparation (3100 units/ml sulphatase and 13000 units/ml ß-glucuronidase from the snail *Helix pomatia*: Sigma, St Louis, MO, USA) to convert sulphated oestrogens into free oestrogens. The samples were extracted 3 times with diethyl ether (4 ml) after the addition of 1000 cpm of [2,4,6,7-^{3}H]oestrone (Radiochemical Centre, Amersham, UK) for estimation of procedural losses. The residues of the diethyl ether extracts were evaporated under a

stream of nitrogen, redissolved in 250 µl toluene:methanol (9:1, v/v) and applied to chromatography columns (8.0 x 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 and oestrone was eluted in the next 3 ml fraction. These E1 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]oestrone (78%). Oestrone concentrations were measured in duplo (2 aliquots of 150 µl) by RIA using a specific rabbit antiserum against 6-keto-oestrone 6-CMO-BSA. The main cross-reacting steroid was oestradiol (0.45%). The antiserum was used in a working dilution of 1:30000. The sensitivity of the assay was 13 pg/ml at the 90% B/B0 level. The intra-assay coefficient of variation was 12.4% and the inter-assay coefficient of variation was 15.7%.

Statistical analysis. Differences in LH and progesterone concentration of pregnant and cyclic gilts were tested for significance after ln transformation of the data by Student's *t* test (SPSS Inc., 1988).

Results

In all gilts the highest LH concentrations were measured 3 h after injecting the GnRH analogue. The LH peaks did not differ between cyclic (Fig. 1) and pregnant gilts (Fig. 2).
In both groups progesterone concentrations increased above 1 ng/ml on Day 2. In the group of the 7 cyclic gilts the highest progesterone concentration was measured on Day 13 (34.8 ng/ml). Thereafter the progesterone concentration declined and returned to the basal level on Day 19 (Fig. 1).
In the group of the 6 gilts pregnant up to Day 35, the highest progesterone concentration was measured on Day 16 (50.8 ng/ml) and progesterone remained at a level of 33 ng/ml (range 27.1-44.4) from Day 19 onwards. From Day 14 there was a significant

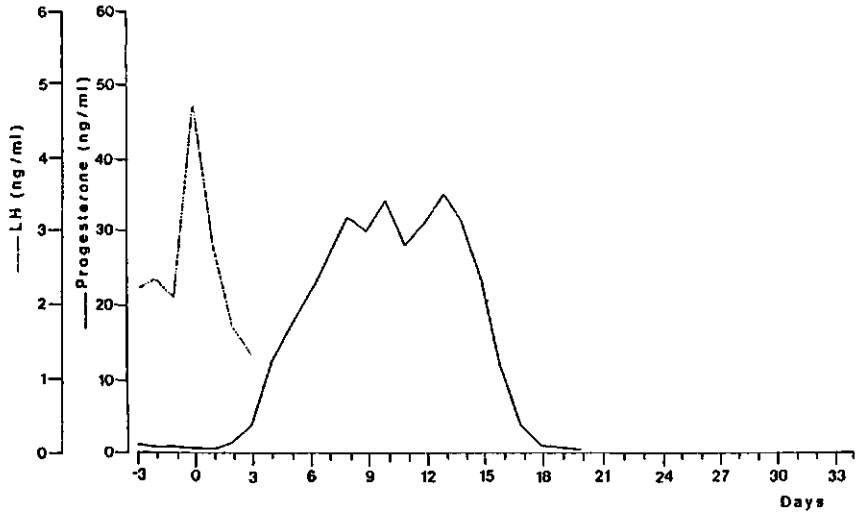

Fig. 1. The LH profile around oestrus and the progesterone profile of 7 cyclic gilts.

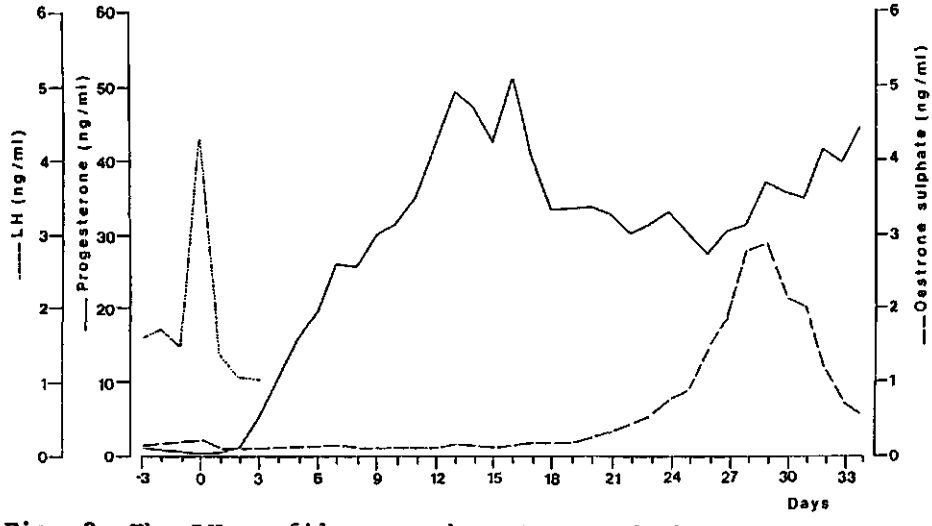

Fig. 2. The LH profile around oestrus and the progesterone and oestrone sulphate profile of 6 pregnant gilts.

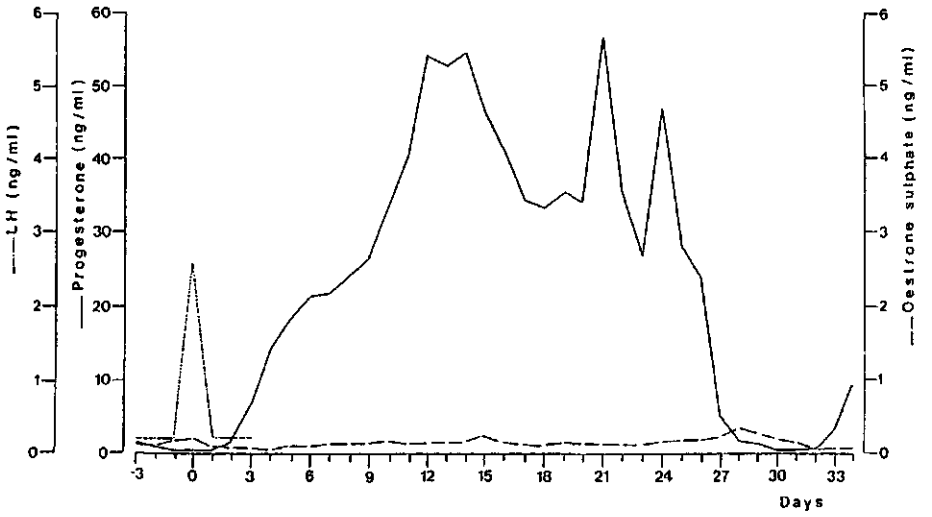

Fig. 3. The LH profile around oestrus and the progesterone and oestrone sulphate profile of Gilt 161, which returned to oestrus on Day 30.

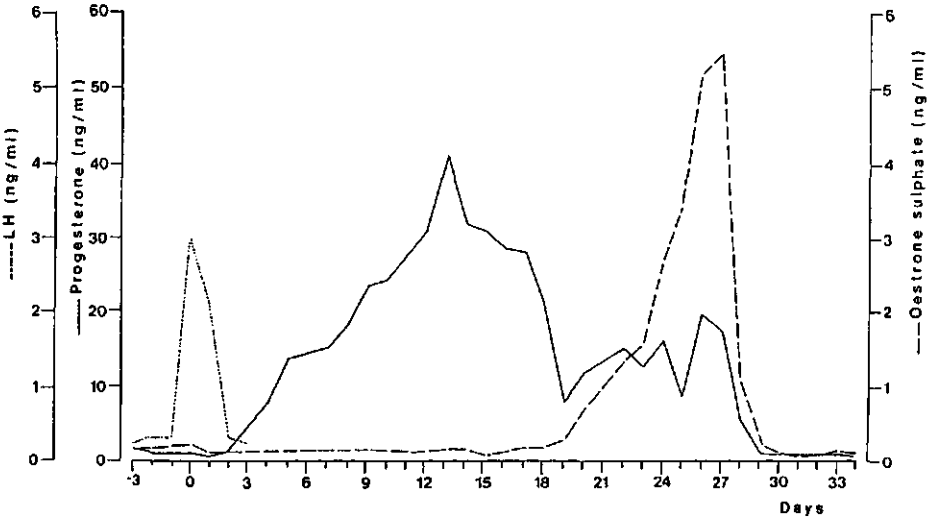

Fig. 4. The LH profile around oestrus and the progesterone and oestrone sulphate profile of Gilt 11, which aborted on Day 28.

difference in progesterone level between the cyclic and pregnant gilts (Day 14 and 15: $P<0.05$; Day 16: $P<0.01$). In pregnant gilts the oestrone sulphate concentration increased from Day 19, reached a maximum on Day 29 (2.9 ng/ml) and declined thereafter (Fig. 2).

In Gilt 161 the progesterone concentration was highest on Day 14 (54.5 ng/ml) and remained at a level of 32.1 ng/ml (range 24.1-34.7) from Day 21 onwards. The oestrone sulphate concentration did not increase but remained at basal levels. On Day 27 the progesterone concentration suddenly declined to basal values. Around Day 28 the oestrone sulphate concentration was slightly increased. The gilt returned to oestrus on Day 30 and the progesterone concentration started to increase again from Day 33 (Fig 3).

In Gilt 11 the progesterone concentration was highest on Day 13 (40.9 ng/ml) and fluctuated between Day 19 to 27 around a level of 14 ng/ml (range 7.6-19.3 ng/ml). From Day 19 the oestrone sulphate level increased to a maximum of 3.8 ng/ml on Day 27. On Day 28 both progesterone and oestrone sulphate concentrations suddenly dropped (Fig. 4). On this day, six aborted macroscopically normal foetuses were found. After the abortion the gilt was not seen in oestrus up to slaughter on Day 35.

Discussion

The progesterone profiles of the cyclic and pregnant gilts are comparable with those reported before (Stabenfeldt *et al.*, 1969; Guthrie *et al.*, 1972; Henricks *et al.*, 1972; Ellendorff *et al.*, 1976; King & Rajamahendran, 1988). The oestrone sulphate profiles of the pregnant gilts also correspond with earlier investigations (Robertson & King, 1974; Horne *et al.*, 1983; Hattersley *et al.*, 1980). However, the time of the highest levels of progesterone and oestrone sulphate, as well as the first time that pregnant and cyclic progesterone levels differ, may vary for 1 or 2 days. In this study a significant

difference between the progesterone levels of cyclic and pregnant gilts exists at Day 14. These progesterone profiles indicate that CL regression during pregnancy has to be prevented already before Day 14. This is in agreement with the observation of Dhindsa & Dziuk (1968) that blastocysts must be present in both uterine horns between Days 10 and 12 for continuation of CL function.

In commercial units there are 2 distinct periods of returning to oestrus after service (Glossop & Foulkes, 1988). The major part of the sows returns to oestrus on Day 20.7±1.4 (range 17-23), and a second group (25.6%) returns on Day 26.5±2.3 (range 24-31). In this study Gilt 161 returned to oestrus during the second period on Day 30. This gilt did not show an increase in oestrone sulphate concentration. This suggests that it may have been pregnant until Day 13, since in gilts in which the blastocysts were flushed out of both horns on Days 12-13 and which returned to oestrus on Day 28.4±2.6 (Van der Meulen *et al.*, 1988), oestrone sulphate concentrations were also not elevated (J. van der Meulen, unpublished data). The blastocysts of this gilt may have generated a first signal for the maintenance of CL function, but they have probably failed to generate a second signal on Days 14-16 (Geisert *et al.*, 1987).

In Gilt 11, in contrast to Gilt 161, the oestrone sulphate concentration increased from Day 19 to 27 and did not deviate from that of pregnant gilts. From Day 19 the progesterone level of this gilt varied around 14 ng/ml, which is relatively low compared with the progesterone concentration of pregnant gilts, but still higher than the minimum daily progesterone requirement for maintenance of pregnancy (Ellicott & Dziuk, 1973). The normal oestrone sulphate concentration and the normal appearence of the aborted foetuses indicate a normal embryonic development until Day 27. Although an embryonic malfunction cannot be excluded, the relatively low progesterone level and the failure to return to oestrus after abortion suggest a maternal cause for pregnancy failure in this gilt.

The data of Gilt 161 support the suggestion of Glossup & Foulkes (1988) that return to oestrus after service on Days 24-31 is caused by embryonic loss. This embryonic loss may already occur around Day 14-16 after a first signal for CL rescue has been generated.

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Chapter IV

Effect of flushing of blastocysts on Days 10-13 on the life-span of the corpora lutea in the pig

J. van der Meulen, F.A. Helmond and C.P.J. Oudenaarden

Department of Animal Physiology, Agricultural University, Haarweg 10, 6709 PJ Wageningen, The Netherlands

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Summary

Blastocysts were flushed out of both uterine horns of gilts on Days 10, 11, 12 or 13. In mated non-pregnant gilts flushing had no effect on progesterone profile or cycle length (20.8±0.4 versus 20.6±0.6 days in the preflush cycle, N=6, mean ± s.e.m.). Flushing the blastocysts out of the uterine horns on Day 10 resulted in a cycle with a normal progesterone profile and a normal length (21.2±0.4 days, N=5). Flushing on Days 11, 12 or 13 resulted in a normal cycle or in maintenance of the CL for 3-13 days as indicated by elevated progesterone concentrations and an increased interoestrous interval of, respectively, 22.0±1.2 versus 19.8±0.6 days (Day 11; N=6), 24.8±1.4 versus 21.0±0.6 days (Day 12; N=5; $P<0.05$) and 26.3±2.3 versus 20.5±0.4 days (Day 13; N=6; $P<0.05$). There was a positive relationship between the change in interoestrous interval and the interval between the first observed standing oestrus and flushing of the blastocysts ($r_s=0.350$; n=22; $P<0.1$). There was a large variation in the diameter of the blastocysts flushed on the same day. Only in those gilts in which the blastocysts were ≥8 mm or filamentous were the CL maintained for 3 or more days. These results indicate that a first signal for maternal recognition of pregnancy is generated on Day 12 and that blastocysts ≥8 mm are required for prolongation of CL function for 3 or more days. Since CL function is only extended for a maximum of 13 days (mean 7.4±1.0), a second signal seems necessary to maintain the CL for the whole period of pregnancy.

Introduction

The establishment and maintenance of pregnancy includes the exchange of signals between the developing conceptus and the maternal system. After fertilization and development to the blastocyst stage, the conceptus must provide a signal to bring about the maintenance of the corpora lutea (CL) and the

continuation of progesterone secretion. The prolongation of the functional life-span of the CL is a reflection of the maternal recognition of pregnancy which has occurred.

There is a critical period during which the blastocysts must provide the signal responsible for the maternal recognition of pregnancy. So far, this period has only been shown in experiments with unilaterally pregnant gilts. Removing blastocysts from one uterine horn on Day 12 or later does not disturb pregnancy in the other horn, whereas pregnancy is interrupted if blastocysts are flushed out of one horn on Day 4 or 10, i.e. blastocysts must be present in both uterine horns between Days 10 and 12 for continuation of pregnancy, while blastocysts need to be present in one horn only after Day 12 (Dhindsa & Dziuk, 1968).

Other evidence about the time period in which the maternal recognition of pregnancy takes place involves administration of exogenous oestrogens, since oestrogens appear to provide the signal for the maternal recognition of pregnancy in the pig (Bazer & Thatcher, 1977; see, for review, Bazer *et al.*, 1984). Daily i.m. or intrauterine injections of oestradiol valerate or benzoate on Days 11-15 in non-pregnant gilts prolong luteal function for a variable time (Kraeling *et al.*, 1975; Frank *et al.*, 1977, 1978; Bazer *et al.*, 1982; Ford *et al.*, 1982b; Saunders *et al.*, 1983; Ziecik *et al.*, 1986; Geisert *et al.*, 1987). One single i.m. injection of oestradiol benzoate between Days 9.5 and 14 results in maintenance of the CL for approximately 7-10 days (Kidder *et al.*, 1955; Garverick *et al.*, 1982; Geisert *et al.*, 1987). However, no change in interoestrous interval occurs with administration before Day 9 or after Day 16 (Kidder *et al.*, 1955; Geisert *et al.*, 1987).

In this study the time of the maternal recognition of pregnancy was determined in a more direct way by flushing the blastocysts out of both uterine horns of pregnant gilts. It was investigated whether a relationship exists between the time of flushing or the blastocyst size and the life-span of the CL.

Materials and Methods

Animals and experimental design. Twenty-seven crossbred gilts (Great Yorkshire x Dutch Landrace) which had shown 2 or more normal oestrous cycles (18-22 days) were used. The gilts were checked for oestrus with a vasectomized boar twice daily (09:00 and 15:00 h). At least 5 days before expected oestrus for the treatment cycle, they were fitted with an indwelling jugular vein catheter (PVC, 1.0 mm i.d., 1.5 mm o.d.). Blood samples were collected 3 times a day (09:00, 12:00 and 15:00 h). At the time of observing first standing heat (Day 0) 100 µg GnRH analogue (Ovalyse: Upjohn Company, Ede, The Netherlands) were injected i.m. and artificial insemination (AI) was carried out on Day 1, about 26 h after GnRH injection (Van der Meulen *et al.*, 1986). Blastocysts were flushed out of the uterine horns of the gilts at surgery on Day 10 (N=5), Day 11 (N=6), Day 12 (N=5) or Day 13 (N=6). Mated non-pregnant gilts were flushed on Days 10, 11, 12 or 13 (Controls, N=5). Blood sampling and checking for oestrus continued until slaughter 34 days after AI.

Surgery. The left uterine horn was exposed by lateral laparotomy under general anaesthesia with metomidate-azaperone (Hypnodil-Stresnil: Janssen Pharmaceutica, Beerse, Belgium). The corpora lutea were marked with India ink to indicate at slaughter whether regression had taken place. A small incision was made just below the utero-tubal junction. A catheter was then passed through the incision into the uterine lumen and the cuff was inflated. A forceps was placed at the utero-cervical junction and a second catheter was placed just before this junction as described above. Through the first catheter 40 ml sterile saline (0.9 % NaCl) were injected into the uterine lumen. Together with the fluid the blastocysts were forced by hand pressure towards the cervix and out through the second catheter into a sterile bottle. This was repeated with 20 ml flushings until blastocysts were no longer in the flushings. After removing the catheters and suturing the

incisions, the right uterine horn was treated in the same way. The blastocysts were measured and classified according to Pope & First (1985).

Hormone analysis. All blood samples were collected into heparinized tubes and centrifuged; plasma was stored at -20°C until further analysis.

Plasma concentrations of progesterone were measured in all samples by RIA (Helmond *et al.*, 1980), using a specific rabbit antiserum against 4-pregnene-6ß-ol-3,20-dione-hemisuccinate-BSA. The main cross-reacting steroids were pregnenolone (98.0%), corticosterone (2.7%), 17α- hydroxyprogesterone (1.5%) and 20α-hydroxy-4-pregnen-3-one, cortisol, oestrone, oestradiol-17ß, oestradiol-17α, oestriol, androstenedione, dehydroandrosterone and testosterone (all <0.2%). The antiserum was used in a working dilution of 1:15000. The sensitivity of the assay was 0.1 ng/ml at the 90% B/B_0 level. The intra-assay coefficient of variation was 7.1% and the inter-assay coefficient of variation was 15.6%.

Plasma concentrations of LH were measured in the samples collected around oestrus by a double-antibody RIA (Niswender *et al.*, 1970), using pig LH (LER 786-3, potency 0.65 x NIH-LH-S1) as a standard and for iodination. Anti-ovine LH 614 IV was used at a 1:30000 working dilution and goat anti-rabbit immunoglobulin as the second antibody. The sensitivity of the assay was 0.7 ng/ml at the 90% B/B_0 level. The intra-assay coefficient of variation was 13.8% and the inter-assay coefficient of variation was 14.8%.

Statistical analysis. The results for length of preflush and flush cycle were analysed by Student's paired *t* test (two-tailed). Spearman rank correlation coefficient was calculated between the time interval of the first observed standing oestrus and flushing on the one hand and the change in interoestrous interval on the other (Snedecor & Cochran, 1980). All data are expressed as mean ± s.e.m.

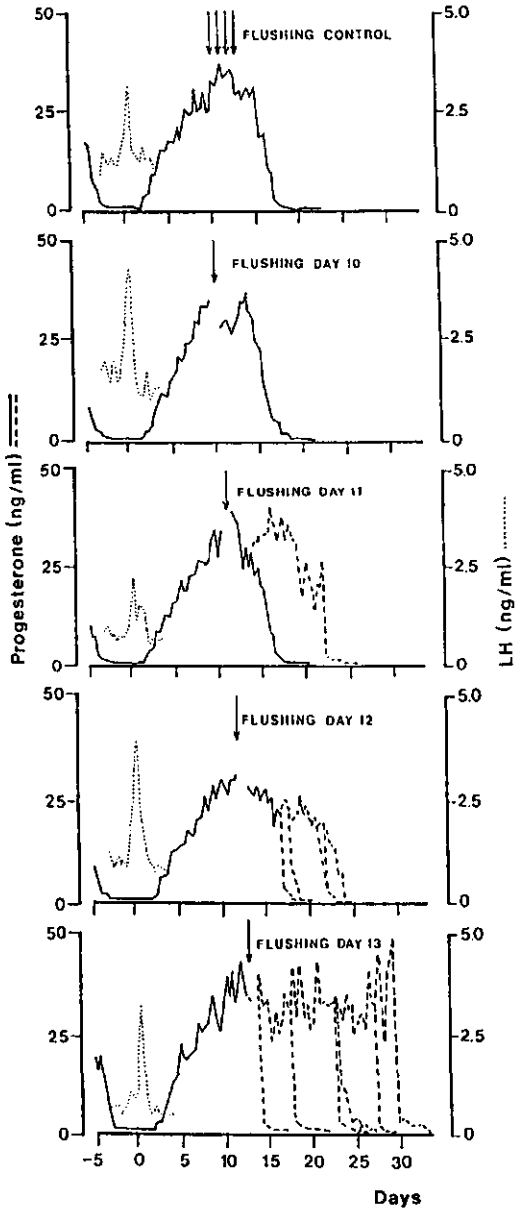

Fig. 1. The LH profile around oestrus and the progesterone profile before and after flushing until the next oestrus of mated non-pregnant gilts and gilts flushed (arrows) on Day 10, 11, 12 and 13. When progesterone concentrations did not return to basal values around Day 18, the progesterone values of individual gilts are depicted (broken lines).

Results

In all five groups the highest LH concentrations were found 3 h after injecting the GnRH analogue. In all mated non-pregnant gilts and Day-10 flushed gilts, plasma progesterone concentrations declined to basal values between Days 15 and 18 (Fig. 1). In the other groups, plasma progesterone concentrations remained elevated after Day 18 in at least some gilts (Fig. 1). In one Day-13 flushed gilt, progesterone concentration did not decline until Day 30, but at slaughter on Day 35 the marked CL of this and all the other gilts had undergone regression. Compared with the duration of the preflush cycle (which did not differ for the 5 groups) the interoestrous interval of the gilts with extended luteal function had increased by 4-13 days (mean 7.4±1.0, N=8). This increase was significant for Day-12 flushing (21.0±0.6 versus 24.8±1.4 days, $P<0.05$) and Day-13 flushing (20.5±0.4 versus 26.3±2.3 days, $P<0.05$). Compared with flushing of mated non-pregnant gilts and Day-10 pregnant gilts the interoestrous interval after flushing on Day 11 was not significantly different, but after flushing on Day 12 and Day 13 the interoestrous interval was significantly longer (both $P<0.05$) (Table 1).

In the gilts in which flushing did not change the interoestrous interval for more than 3 days, the maximal blastocyst size varied from 0.5 to 6.9 mm in 11 gilts and in 3 gilts the blastocysts were filamentous. In the gilts with an increased interoestrous interval, after flushing of at least 3 days, the blastocysts were at least 8.0 mm or filamentous.

In all the gilts the time interval between the first observed standing heat and flushing ranged from 240 to 321 h. There was a positive relationship between this time interval and the increase in the interoestrous interval ($r_s=0.350$; n=22; $P<0.1$).

Table 1. Interoestrous interval of preflush cycles and cycles when blastocysts were flushed from both uterine horns of gilts on different days.

Day of flushing	No. of gilts	Cycle length (days)		No. of gilts with a change in cycle length of >3 days
		Preflush	Flush	
control	5	20.6±0.6	20.8±0.4	0
10	5	20.6±0.2	21.2±0.4	0
11	6	19.8±0.6	22.0±1.2	1
12	5	21.0±0.6[a]	24.8±1.4[b]	3
13	6	20.5±0.4[a]	26.3±2.3[b]	4

Values with different superscripts are different ($P<0.05$).

Discussion

Since the cycle length may vary by about 2 days (Andersson & Einarsson, 1980; own observations), an increase in the interoestrous interval of at least 3 days is considered as an extension of the CL function. The results of this study show that blastocysts from Day 11 (just 1 of 6 gilts) or 12 signal their presence in such a way that an extension of the CL function occurs. The correlation between the time interval of the first observed standing heat and flushing on the one hand and the change in interoestrous interval on the other may indicate that the maternal recognition of pregnancy is a continuous process. Prolonged CL function, however, is only established in gilts with spherical blastocysts of ≥8 mm in diameter or filamentous blastocysts. The fact that large spherical blastocysts, just before elongation, already have the ability to prolong CL function finds support in studies concerning both the onset of the production of oestrogens and the presence of oestrogens in uterine flushings of pregnant

animals. The conversion of dehydroepiandrosterone and androstenedione into both oestrone and oestradiol-17ß, *in vitro*, is first detectable in spherical blastocysts of 5 mm (Perry *et al.*, 1976) and the conversion of progesterone into oestradiol is initiated in blastocysts of 7 mm (Fisher *et al.*, 1985). In pregnant sows, on Day 11 the concentration of oestrogens in uterine flushings is elevated, compared with unmated controls, only when the blastocysts are >8 mm (Ford *et al.*, 1982a). Blastocysts of this size also start to secrete low molecular weight acidic proteins, but the function of these proteins is not known (Godkin *et al.*, 1982).

Blastocysts flushed out of both uterine horns on Days 11, 12 or 13 had generated a signal by which the cycle length had increased on the average by 7.5 days with a maximum of 13 days. This indicates that the CL, for the whole duration of pregnancy, require a prolonged signal or a second signal. The increase in cycle length after flushing is largely similar to the one after one intramuscular injection of oestradiol benzoate or diethylstilboestrol on Days 9.5, 11, 12, 12.5 or 14 (Kidder *et al.*, 1955; Garverick *et al.*, 1982; Geisert *et al.*, 1987). The existence of 2 signals is supported by the increase in interoestrous interval for >60 days after a single intramuscular injection of oestradiol benzoate on Day 11 followed by injections on Days 14-16 (Geisert *et al.*, 1987). An increase in interoestrous length, after daily injections of oestradiol benzoate or valerate on Days 11-15, in a few cases to even more than 115 days (Kraeling *et al.*, 1975; Frank *et al.*, 1977, 1978; Bazer *et al.*, 1982; Saunders *et al.*, 1983; Ziecik *et al.*, 1986; Geisert *et al.*, 1987), may also indicate the existence of 2 signals, since intramuscular injection of oestradiol valerate results in elevated plasma oestrogen concentrations for several days after treatment (Geisert *et al.*, 1982). Although oestrogens may account for both signals, the first signal is completed by Day 14, as is indicated by a reduction of oestrogens in uterine flushings on Day 14 to non-pregnant levels (Geisert *et al.*, 1982; Bazer *et al.*, 1984; Stone & Seamark, 1985). In pregnant gilts oestrogens in

uterine flushings start to rise for the second time by Day 15 (Stone & Seamark., 1985) and the second signal may occur around attachment (starting on about Day 16; King *et al.*, 1982), the next crucial step in establishment of pregnancy after elongation (Morgan *et al.*, 1987). Around Day 20, this second signal must have been completed, since intramuscular injection of oestradiol benzoate on Day 11 and on Days 20-22 results in a cycle of normal length in half of the gilts and in an increased interoestrous interval of about 60 days in the rest (Geisert *et al.*, 1987).

Glossop & Foulkes (1988) provide further evidence to support the hypothesis of the existence of 2 signals. In their study 25.6% of the sows returning to service did not do so by Day 21 but by Day 26.5±2.3. This return to oestrus takes place almost at the same time as after flushing of blastocysts on Day 12 or 13 in this study. This indicates that these sows may have been pregnant around Day 13 (supported by the rosette inhibition titre in 2 sows, 2 weeks after mating, but returning to oestrus on Day 26; Morton *et al.*, 1983), and that the blastocysts had been able to generate a first signal for the maternal recognition of pregnancy. In those sows the prolongation of the first signal or the generation of a second signal by the blastocysts seems to have been disturbed. Therefore, additional studies are needed to investigate the role of the 2 signals necessary for CL maintenance for the whole duration of pregnancy in the pig.

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CHAPTER V

Aromatase activity in individual Day-11 pig blastocysts

J. van der Meulen[1], G. te Kronnie[2], R. van Deursen[1] and J. Geelen[3]

[1]Department of Animal Physiology, Agricultural University, Haarweg 10, 6709 PJ Wageningen, The Netherlands;
[2]Department of Experimental Animal Morphology and Cell Biology, Agricultural University, Marijkeweg 40, 6709 PG Wageningen, The Netherlands;
[3]Organon International BV, Scientific Development Group, P.O. Box 20, 5340 BH Oss, The Netherlands

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Summary

Blastocysts were flushed from both uterine horns of 10 gilts on Day 11 of pregnancy. In these gilts 15.1±2.3 (mean ± s.d.) corpora lutea were present and 12.7±3.1 spherical blastocysts were recovered. In all the gilts variation in blastocyst diameter was observed. Aromatase activity was measurable in 118 of 121 examined blastocysts and varied from 0.005 to 19.330 pmol [1ß-^{3}H]androstenedione converted into ^3H_2O in 20 min (mean 1.31). This variation in aromatase activity reflected a difference between and within gilts. Of the total variation between all blastocysts, 67% was due to differences between gilts. A positive exponential relationship existed between blastocyst diameter and aromatase activity, but this relationship was different between gilts (P<0.0001). The observed variation in aromatase activity may be caused by differences in germ layer differentiation of the blastocysts.

Introduction

In the pig oestrogens appear to be responsible for the maternal recognition of pregnancy (Bazer & Thatcher, 1977; Bazer *et al.*, 1984). During the period of maternal recognition of pregnancy, oestrogens increase the bloodflow (Ford *et al.*, 1982) and change the uterine environment by stimulating the release of secretory material from the uterine glandular epithelium (Geisert *et al.*, 1982). The synthesis of oestrogens is already measurable in pooled spherical Day 11-12 blastocysts with an average diameter of 5-7 mm (Perry *et al.*, 1976; Fischer *et al.*, 1985; Mondschein *et al.*, 1985).

Amongst the blastocysts of one litter a considerable variation in diameter can exist on Day 11 of pregnancy (Anderson *et al.*, 1978; Stroband *et al.*, 1984). Pope *et al.* (1982) suggested that the larger blastocysts of such a litter may have a better chance for survival. Such blastocysts are morphologically more developed and their advanced synthesis of

oestrogens may alter the uterine environment in such a way that their less developed littermates can no longer survive (Pope *et al.*, 1982; Pope & First, 1985). Advancing the uterine environment of pregnant gilts on Day 11 by administration of oestradiol valerate on Days 9-10 results in pregnancy failure in about 50% of the treated gilts on Day 16 or 30 (Pope *et al.*, 1986; Morgan *et al.*, 1987). An advanced uterine environment beyond Day 11 did not affect the pregnancy rate (Pope *et al.*, 1986).

The survival of just some of the blastocysts within a litter may be caused by a difference in time of onset of oestrogen release or by the amount of oestrogen released by individual blastocysts of the litter. Although variation in blastocyst diameter within litters has been described (Anderson, 1978; Stroband *et al.*, 1984), nothing is known about the aromatase activity of individual blastocysts.

In this study the aromatase activity of individual Day-11 blastocysts was investigated. The variation in aromatase activity within and between gilts was determined and correlated with variation in blastocyst diameter.

Materials and methods

Animals. Crossbred gilts (Great Yorkshire x Dutch Landrace) were checked for oestrus with a vasectomized boar twice daily (08:00 and 16:00 h). They were artificially inseminated on Day 1, 24 h after the time of observing first standing oestrus (Day 0). On Day 11 of pregnancy the 10 gilts were slaughtered. The blastocysts were recovered within 10 min after death by flushing both uterine horns twice with 30 ml Dulbecco's phosphate-buffered saline (PBS, Gibco, Paisley, UK). The diameters of the blastocysts were measured and the blastocysts were rinsed in Dulbecco's PBS supplemented with dithiotreitol (5 mg/l: Sigma, St Louis, MO, USA). The blastocysts were individually frozen in 100 µl of the last rinsing buffer within 20 min after slaughter and stored at -80°C until

analysis.

Chemicals. [1ß-^{3}H]Androstenedione and En^{3}Hance scintillation spray were purchased from Du Pont de Nemours, Dreieich, FRG; glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP from Boehringer, Mannheim, FRG; EDTA from Baker, Deventer, The Netherlands; BSA from Sigma, St Louis, MO, USA; Dextran T70 from Pharmacia, Uppsala, Sweden; and Norit A and precoated silicagel HF 254 thin-layer plates from Merck, Darmstadt, FRG. Liquid scintillation counting of tritium was done with picofluor-30 (Packard Instrument BV, Groningen, The Netherlands) in a 2200 CA-Tri-CARB liquid scintillation analyser (Packard Instrument Company, Downers Grove, IL, USA).

Aromatase assay. Aromatase activity was determined by measuring the amount ^3H_2O released from [1ß-^{3}H] androstenedione, according to the procedure of Thompson & Siiteri (1974).
The blastocysts were homogenized in an-all glass pestle in potassium phosphate buffer (10 mM, 1mM EDTA, 1 g BSA /l, pH 7.4). The blastocyst homogenate was incubated with [1ß-^{3}H] androstenedione (sp. act. 1.036 TBq/mmol, 90.4 pmol) and an NADPH-generating system (2.5 mM NADP, 5.0 mM glucose 6-phosphate, 525 units glucose 6-phosphate dehydrogenase /l). The reaction was allowed to proceed in air in a shaking water bath for 20 min at 37°C, and terminated by addition of chloroform (5 ml) and thorough mixing. After centrifugation (15000 *g*, 10 min, 20°C) an aliquot of the aqueous phase (1.8 ml) was mixed with 0.2% of a trichloroacetic acid solution (300 g/l distilled water) and centrifuged (15000 *g*, 5 min, 20°C). A part of the supernatant (1 ml) was mixed with an equal volume of Dextran-coated charcoal suspension (50 g Norit A and 5 g Dextran T70 /l distilled water) and centrifuged (15000 *g*, 10 min, 20°C). Then 1.0 ml supernatant was transferred to scintillation vials, 4 ml picofluor-30 were added and ^3H_2O was counted. A part of the chloroform fraction (2.5 ml) together with 0.2 mg androstenedione and testosterone was evaporated

under a stream of nitrogen at 45°C. The residue was dissolved in 200 µl ethanol and a part of this solution was applied to a thin-layer plate. This plate was developed in chloroform-ethylacetate (4:1 v/v, unsaturated chamber), dried, and after spraying with En^{3}Hance spray it was stored with X-Omat RP Film (Kodak, France) at -70°C for several days. After developing the film it could be checked whether the amount of [1ß-^{3}H]androstenedione used had been restrictive for oestrogen production. The intra- and inter-assay coefficients of variation were 1.5% and 7.6%, respectively. The aromatase activity is expressed as the amount of [1ß-^{3}H]androstenedione converted into ^3H_2O in 20 min.

Statistical analysis. The aromatase activity was ln transformed and within-litter regression on diameter was performed using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Inc., 1985). Differences in average blastocyst diameter and average aromatase activity between the uterine horns of individual gilts were tested for significance by Students' *t* test. Data are expressed as mean ± s.d.

Results

In the 10 gilts an average of 15.1±2.3 (range 11-19) corpora lutea (CL) were present. The number of blastocysts recovered ranged from 5 to 16 (average 12.7±3.1). In one gilt (No. 182) the recovery was less than 50%, but in the other gilts the average recovery was 86.7% (Table 1). All the blastocysts recovered were spherical. In all the gilts variation in blastocyst diameter existed. The average difference in diameter between the smallest and largest blastocyst within a litter was 3 mm, and ranged from 1 mm (Gilt 176) to 7 mm (Gilt 121). In 4 gilts there was a significant difference in average diameter between the blastocysts of the left and right uterine horn (Table 1).

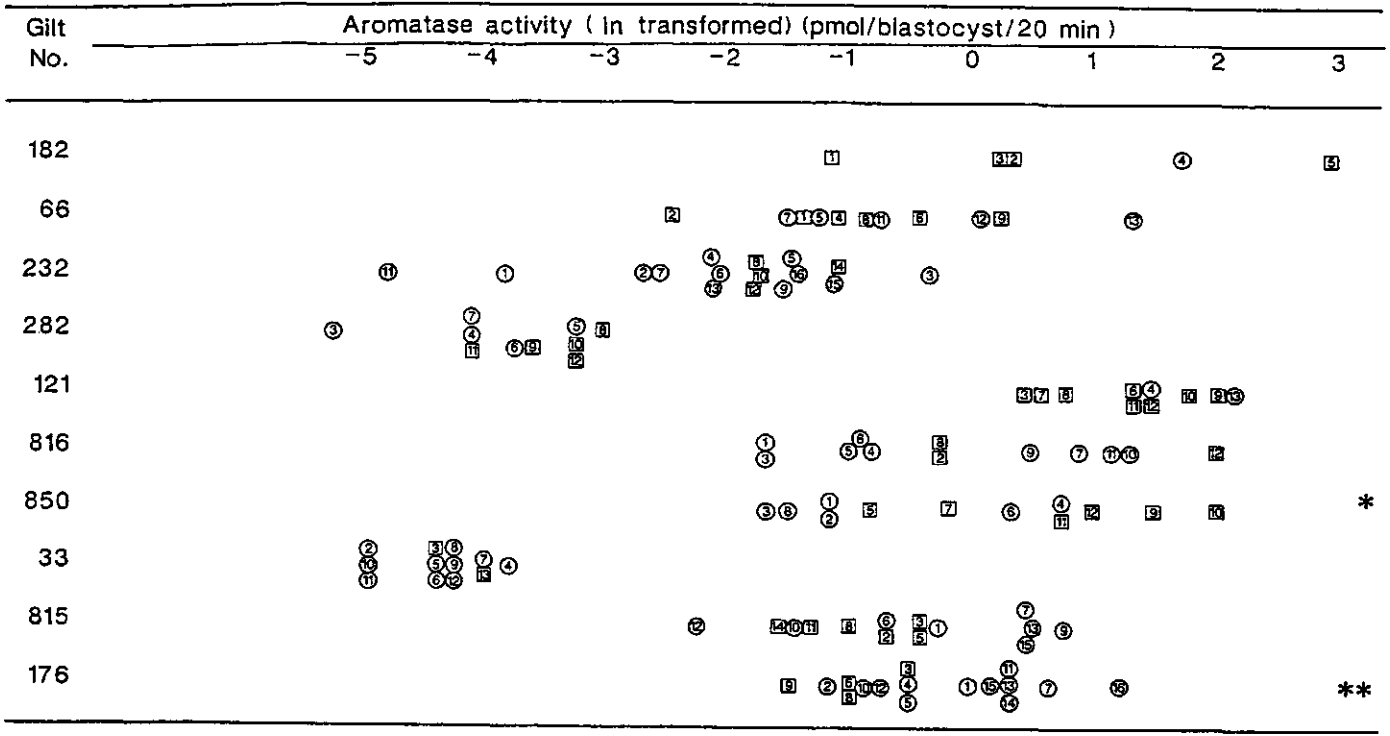
Table 1. The number of CL and blastocysts of 10 gilts slaughtered on Day-11 and the size* of their 127 blastocysts

Gilt No.	CL L	CL R	Blastocysts L	Blastocysts R	Blastocyst diameter (mm) 1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	
182	5	6	1	4						①	②③			④	⑤					
66	2	15	7	6					①		②	③④	⑤⑥⑦⑧	⑨	⑩⑪	⑫		⑬		*
232	9	6	12	4	①				②③④	⑤⑥⑦	⑧⑨⑩⑪⑫⑬⑭	⑮⑯								
282	12	7	7	5	①	②	③④	⑤	⑥⑦	⑧⑨	⑩⑪	⑫								**
121	11	3	2	11	①					②		③		④⑤	⑥⑦⑧	⑨⑩	⑪⑫		⑬	
816	11	4	8	4							①②	③④⑤⑥	⑦	⑧⑨	⑩	⑪				
850	8	6	6	6						①②	③	④⑤	⑥⑦⑧	⑨	⑩⑪	⑫				**
33	5	8	10	3			①	②	③	④	⑤	⑥⑦⑧⑨	⑩⑪	⑫		⑬				
815	12	5	8	7						①②③④	⑤	⑥⑦⑧⑨⑩⑪⑫⑬⑭			⑮					
176	9	7	12	4						①②③④⑤⑥⑦⑧⑨	⑩⑪⑫	⑬⑭⑮⑯								**

○○ Left horn ; □□ right horn; ○□ : the aromatase assay of these blastocysts failed.
Significant difference in blastocyst diameter between left and right horn: * $P < 0.05$; ** $P < 0.01$.

* The blastocysts of each gilt were numbered according to increasing size.

Table 2. The aromatase activity of 118* Day-11 blastocysts** (ln transformed)



○ Left horn ; □ right horn.

Significant difference in aromatase activity between left and right horn: * $P < 0.05$, ** $P < 0.01$.

* Aromatase activity determination failed in 6 blastocysts and was <0.005 pmol/blastocyst/20 min in 3 blastocysts.

** The numbers of the blastocysts correspond to the numbers of Table 1.

Aromatase activity was measurable in all but 3 Day-11 blastocysts (average 1.31 pmol/blastocyst/20 min; range 0.005-19.33). Fluorographs from thin-layer chromatography showed, for all blastocysts in which aromatase activity was assessed, that the amount of [1ß-^{3}H]androstenedione added to the reaction medium had not been limiting. Aromatase activity varied between and within gilts: of the total variation between all blastocysts, 67% was due to differences between gilts. Within gilts aromatase activity was different for blastocysts with the same diameter (Tables 1 and 2, e.g. Gilts 815 and 176), and blastocysts with the same aromatase activity differed in diameter (Tables 1 and 2, e.g. Gilts 282 and 33). In 2 gilts there was a significant difference in average aromatase activity between the blastocysts of the 2 uterine horns (Table 2).

The relationship between aromatase activity and diameter within litters was significantly different for the 10 gilts (R^2=0.88, P<0.0001)(Fig. 1). Even between gilts with almost the same range in blastocyst diameter a large difference in aromatase activity was present (Fig. 1, Gilts 850 and 815).

Discussion

For the first time the aromatase activity of individual spherical pig blastocysts has been measured. All but 3 of 121 blastocysts examined from 10 gilts on Day 11 of pregnancy showed aromatase activity, varying from 0.005 to 19.33 pmol/blastocyst/20 min. This reflects a variation between gilts (67% of the total variation), as well as a variation within gilts (33% of the total variation).

Assuming that differences in aromatase activity reflect differences in developmental stage among the blastocysts it could be an important functional marker to quantitate developmental variation. A commonly used measure to describe variation in blastocyst development in pigs is the diameter of the spherical blastocyst. The results of this study confirm

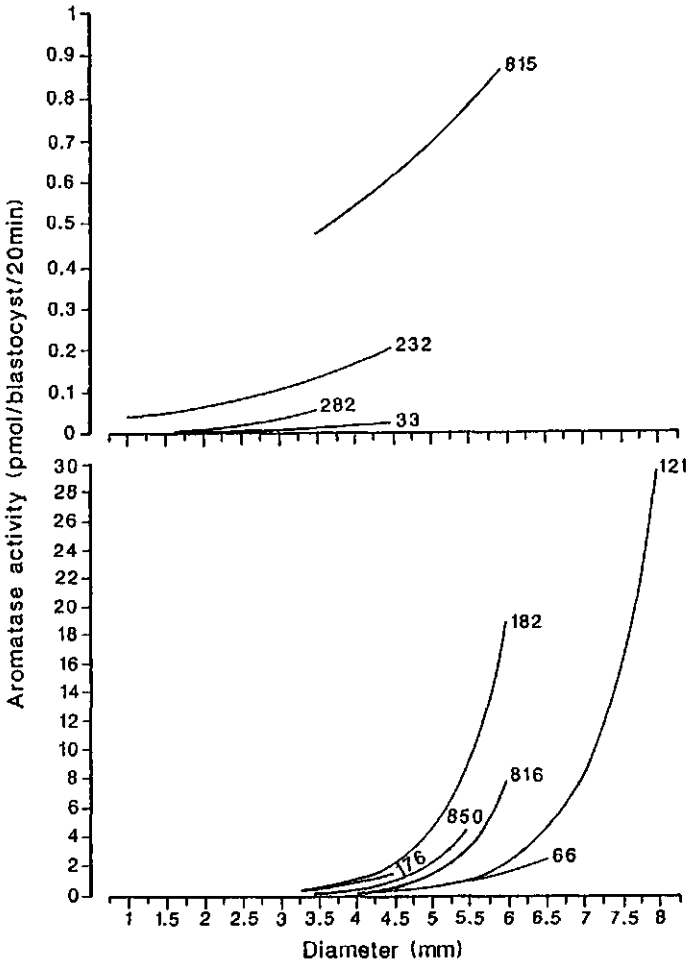


Fig. 1. The relationship between aromatase activity and blastocyst diameter in the 10 gilts (indicated by the no. given).

the presence of variation in Day-11 blastocyst diameter (Anderson, 1978; Stroband *et al.*, 1984). An increase in average blastocyst diameter results in an increase in oestrogen content in uterine flushings from Day 10.5 to 12 (Geisert *et al.*, 1982) and an increase in aromatase activity

in vitro from Day 10 to 13 (Mondschein *et al.*, 1985). The results of this study show that aromatase activity of individual blastocysts differs. On average the aromatase activity of larger blastocysts is higher than in smaller blastocysts; a positive exponential relationship between blastocyst diameter and aromatase activity was observed. Aromatase activity of blastocysts of virtually the same diameter, however, can be different. Embryoblasts of Day-11 blastocysts with nearly the same diameter may also differ greatly in cell number (Te Kronnie *et al.*, 1988). Apparently diameter and aromatase activity or embryoblast cell number do not give the same information about developmental stage.
On Day 10 oestrone has been demonstrated in trophoblast cells (King & Ackerley, 1985), and from Day 12 oestrone and oestradiol-17ß are not only present in trophoblast cells, but also in large quantity in the hypoblast (King & Ackerley, 1985; Bate & King, 1988). The presence from Day 11 onwards of mitochondria resembling those in steroid-producing cells indicates that the ability to synthesize oestrogen is present, not only in trophoblast and hypoblast, but also in embryoblast cells (Stroband *et al.*, 1984). This suggests that aromatase activity depends on the degree of germ layer differentiation. A different degree of germ layer differentiation between blastocysts of the same age may therefore account for the observed difference in aromatase activity between blastocysts of the same age and diameter.

Developmental variation between Day-11 blastocysts may be caused by differences between gilts in the moment of ovulation during oestrus (Stroband *et al.*, 1984; Helmond *et al.*, 1986) and differences in the duration of ovulation (Elze *et al.*, 1987). A genetic paternal influence which has been found in pigs has been suggested as another source of difference in oestrogen production between litters (L.A. Bate & R.R. Hacker, unpublished data, quoted by Bate & King, 1988).

The results of this study, with regard to the existence of differences in aromatase activity between individual blastocysts within an uterus, support the hypothesis of Pope

et al. (1982). To what extent this results in an altered local uterine environment, detrimental for some blastocysts, remains to be investigated. It will be necessary to study the degree of differences in aromatase activity between individual blastocysts and the distribution of the blastocysts within the uterine horns.

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CHAPTER VI

In vitro culture of Day-11 spherical and Day-13 filamentous pig blastocysts without supplementation of oestrogen precursors: ultrastructure and oestrogen release

J. van der Meulen[1], G. te Kronnie[2], C.P.J. Oudenaarden[1] and F.A. Helmond[1]

[1]Department of Animal Physiology, Agricultural University, Haarweg 10, 6709 PJ Wageningen, The Netherlands;
[2]Department of Experimental Animal Morphology and Cell Biology, Agricultural University, Marijkeweg 40, 6709 PG Wageningen, The Netherlands

submitted for publication

Summary

Day-11 spherical and Day-13 filamentous blastocysts were cultured *in vitro* in 4 ml MEM 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-17ß (E2) were released during the first and second 24 h culture period, and this oestrogen release was maximal during the first 24 h in cultures without endometrial tissue. Clewed Day-13 filamentous blastocysts, recovered after flushing, formed trophospheres and also in these cultures both free and conjugated E1 and E2 were released. Oestrogen release was not affected by the presence of endometrial tissue in these cultures. 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 and 9.7 ng E2 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 smaller amounts of conjugated E1 and E2 during *in vitro* culture. During culture no developmental changes occurred in blastocysts and uterine epithelium. Since no precursors were added to the medium, it is suggested that blastocysts synthesize oestrogens from endogenous precursors.

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 $PGF_{2\alpha}$ release (Bazer & Thatcher, 1977).

In vitro Day-11 blastocysts are able to synthesize oestrone (E1) and oestradiol-17ß (E2) from labelled androstenedione and progesterone (Perry *et al.*, 1976; Flint *et al.*, 1979; Mondschein *et al.*, 1985; Fischer *et al.*, 1985). Conversion of pregnenolone, dehydroepiandrosterone and testosterone into oestrogens has been described for Day 14-18 blastocysts (Perry *et al.*, 1973; Gadsby *et al.*, 1980).

The nature of the oestrogen precursor *in vivo* is unknown. Maternal progesterone may be the precursor, since injection of labelled progesterone into the uterine artery on Day 22 of pregnancy results within 3 h in the appearance of labelled oestrogens in foetal membranes and endometrium (Flint *et al.*, 1979). However, cultures of Day-16 trophoblast explants show that progesterone can be synthesized *de novo* (Heap *et al.*, 1981). Also unconjugated oestrogens were synthesized in these cultures even without supplementation of acetate and cholesterol, although their production decreased after 24 h. This indicates that Day-16 trophoblast explants probably synthesize oestrogens from endogenous precursors (Heap *et al.*, 1981). Experiments with ovariectomized gilts treated with medroxyprogesterone acetate to maintain pregnancy, also indicate that trophoblast oestrogens are produced by *de novo* synthesis rather than from circulating progesterone (Flint *et al.*, 1979).

In this study 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 in medium without supplementation of precursors, was investigated. The ultrastructural features of blastocysts and uterine luminal epithelium after culture were examined.

Materials and methods

Animals. Crossbred gilts (Great Yorkshire x Dutch Landrace), which had shown 2 or more normal oestrous cycles (18-22 days), were checked for oestrus 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 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: Sigma, St Louis, MO, USA), streptomycine (400 µg/ml: 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 ligth microscopy.

Oestrogen assay. In each culture total and free E1 and E2 were measured after extraction and column chromatography by RIA, according to 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) and ß-glucuronidase (130000 units/ml) 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 evaporated under a stream of nitrogen, redissolved in 250 µl toluene: methanol (9:1, v/v) and applied to chromatography columns (8.0 x 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

respectively [^{3}H]E1 (78%) and [3H]E2 (72%).
E1 concentration was measured in duplo (2 aliquots of 150 µl) by RIA using a specific rabbit antiserum against 6-keto-oestrone 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 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 duplo (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 oestrone (1.49%) and oestriol (0.21%). The antiserum was used in a working dilution of 1:30000. The sensitivity of the assay

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Figs. 1-2. General morphology of trophospheres formed by Day-13 filamentous blastocysts *in vitro*. T=trophoblast; H=hypoblast; Ts=trophosphere.

Fig. 1. Overview of clewed filamentous blastocysts with trophospheres after 48h of culture, x 25.

Fig. 2. Cross-section of the bilaminar wall of a trophosphere, composed of trophoblast and hypoblast, x 600.

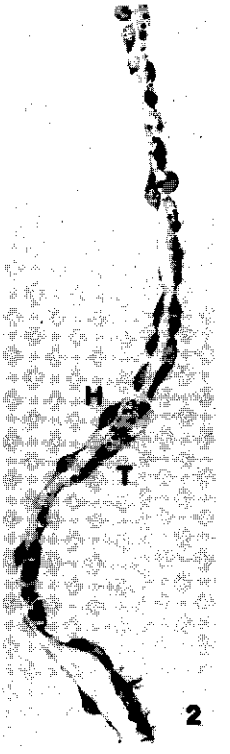
Figs. 3-5. Ultrastructural details of uterine luminal epithelial cells. D=dark granules; Bm=basement membrane; V=secretory vesicles.

Fig. 3. Transmission electron micrograph (TEM) of secretory vesicles under the luminal membrane of endometrial tissue collected on Day-11 before *in vitro* culture, x 21000.

Fig. 4. TEM of secretory vesicles under the luminal membrane of endometrial tissue collected on Day 11 after 48 h *in vitro* culture, x 21000.

Fig. 5. TEM of dark granules at the basal side of the cells of endometrial tissue collected on Day-13 after 48 h *in vitro* culture, x 21000.


Ts


H
T
2


D
Bm
5

was 14 pg/ml 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 amounts of E1 and E2 are expressed in ng/24 h 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 number of blastocysts and their size did not differ between 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 (Figs. 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 (Fig. 1). The trophospheres were composed of flat trophoblast cells and the inside was usually furnished with a network of hypoblast cells (Fig. 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, the 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 (Fig. 5).

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/24 h) free	E1 (ng/24 h) total	E2 (ng/24 h) free	E2 (ng/24 h) 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

In cultures of Day-11 blastocysts, free and conjugated Eland E2 were released (Table 1), but this release was significantly lower during the second 24 h culture period ($P<0.05$). The release of free E1 and E2 was significantly larger during both

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/24 h/blastocyst)		E2 (ng/24 h/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 ($P<0.05$)

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 ($P<0.05$).
In cultures with endometrial tissue, Day-13 clewed filamentous blastocysts released significantly more oestrogens than Day-11 blastocysts ($P<0.05$), but there were no significant differences in oestrogen release by Day-11 and Day-13 blastocysts cultured without endometrial tissue.
Each individually cultured Day-13 filamentous blastocyst released free and conjugated E1 and E2. Release of free E1 and E2 was lower in the second culture period than in the first 24 h culture period ($P<0.05$). During both the first and the second culture period, significant differences in oestrogen release existed between the gilts (Table 2). During the first 24 h on average 2.6 ng/blastocyst E1 and 9.7 ng/blastocyst E2 were released.

Discussion

During culture the spherical blastocysts did not elongate while their gross morphology preserved. Also the endometrial tissue did not seem to develop under the *in vitro* conditions. The observation of secretory vesicles at the luminal side of the uterine epithelium on Day 11 which did not alter during subsequent culture for 48 h, is in contrast with the disappearance of these vesicles around Day 11 *in vivo* (Stroband *et al.*, 1986). Pig blastocyst elongation *in vitro*, which has not been reported sofar, apparently is not facilitated by the endometrial tissue co-culture conditions used in this study. Cellular alterations in uterine epithelium from Day 11 to 13 may be crucial for blastocyst elongation. An endometrium culture system which mimics these cellular alterations may be advantageous for pig blastocyst development *in vitro*.

The trophospheres formed by Day-13 filamentous blastocysts are comparable to those formed by Day 14-18 dispersed blastocyst tissue (Whyte *et al.*, 1986-1987). In this study, however, not all trophospheres were of bilaminar composition. It is assumed that the hypoblast is absent in those cases in which only one layer is observed. Formation of trophospheres does not seem to depend on a complete lining of the trophoblast by the hypoblast and is not dependent on the presence of endometrial tissue in culture. Uterine epithelial cells at Day 13 contain dark granules at the base 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 during culture, analysis of the culture media showed secretion of oestrogens. Both Day-11 spherical and Day-13 filamentous blastocysts released 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.*, 1981). 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 have endogenous precursors which are converted to oestrogens. Decrease in oestrogen release during the second culture period 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 this study a significantly larger release of E2 compared with E1 was 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 into E2 compared with E1 by homogenized filamentous Day-13 blastocysts (J. van der Meulen, G. te Kronnie, R. van Deursen & J. Geelen, unpublished data). In uterine flushings of pregnant gilts on Days 10-13 also more E2 than E1 is measured (Zavy *et al.*, 1980; Geisert *et al.*, 1982).

The differences observed 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 sulphation of blastocyst free oestrogens (Geisert *et al.*, 1982) by sulphotransferase 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 14.9, 20.3, and 14.8% of the initial amount of labelled dehydroepiandrosterone, androstenedione and testosterone, respectively, in the aqueous fraction containing steroid conjugates, sulphates and glucuronides. Conversion of labelled androstenedione into conjugated oestrogens has also been measured in cultures of trophospheres of dispersed Day 14-18 blastocysts (Whyte *et al.*, 1986-1987), and conjugated oestrone 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 significantly negatively affected by the presence of endometrial tissue. Whether this depends on an altered oestrogen release by the blastocysts or an increased uptake by the endometrium remains to be investigated.

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 measured release during *in vitro* culture showed that filamentous Day-13 blastocysts released on average 2.6 ng E1 and 9.7 ng E2 in 24 h. These data and the amounts of E1 and E2 measured in uterine flushings (Zavy *et al.*, 1980; Geisert *et al.*, 1982), indicate that the dose of oestradiol valerate or oestradiol benzoate administered to evoke pseudopregnancy (Bazer *et al.*, 1982) by far exceeds physiological quantities.

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. This release of oestrogens was not accompanied by development of blastocysts and endometrium. Since no precursors were added to the medium, it is suggested that blastocysts synthesize oestrogens from endogenous precursors.

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Chapter VII

Oestradiol receptors in endometrial cytosol of gilts on Days 10-13 of oestrous cycle and pregnancy

J. van der Meulen, F.A. Helmond and C.P.J. Oudenaarden

Department of Animal Physiology, Agricultural University,
Haarweg 10, 6709 PJ Wageningen, The Netherlands

submitted for publication

Summary

Cytosolic oestradiol receptors (ER_c) were determined with a Dextran-coated charcoal assay and Scatchard plot analysis in endometrial tissue of non-pregnant and pregnant gilts on Days 10-13. The Kd of the ER_c was on average 0.40 nM (range 0.09-1.50) and was not affected by day or reproductive state. The endometrial ER_c concentration was affected by day and reproductive state ($P<0.001$). During pregnancy, the endometrial ER_c concentration declined from Day 10 to 13, but did not change in non-pregnant gilts. On Day 12 of pregnancy, the endometrial ER_c concentration was significantly larger in gilts with spherical blastocysts than in gilts with filamentous blastocysts ($P<0.01$).

Introduction

In the pig oestrogens from blastocyst origin are supposed to be responsible for the maternal recognition of pregnancy. The oestrogens alter the direction of $PGF_{2\alpha}$ secretion by the uterine endometrium, and in this way $PGF_{2\alpha}$ is prevented to enter the uterine vasculature and exert its luteolytic effect on the corpora lutea (Bazer & Thatcher, 1977). *In vitro* oestrogen synthesis is started by spherical blastocysts on Day 11 (Perry *et al.*, 1976; Fischer *et al.*, 1985; Mondschein *et al.*, 1985), and *in vivo* prolongation of corpus luteum function is achieved from Day 12 by filamentous and spherical blastocysts ≥8 mm (Van der Meulen *et al.*, 1988).

Ovarian steroid secretion is important in regulating oestrogen receptor concentration in uterine endometrium (Deaver & Guthrie, 1980). During the period of the maternal recognition of pregnancy, oestrogen release by blastocysts within the uterine horns may interact with the endometrial oestrogen receptor and thereby mediate the anti-luteolytic effect (Deaver & Guthrie, 1980).

So far, no study has focussed on endometrial oestradiol receptor concentrations around the time of maternal recognition of pregnancy, and the data available are partly contradictory. In this study oestradiol receptor concentrations in endometrial cytosol of non-pregnant and pregnant gilts were measured on Days 10-13 when a first signal for CL maintenance is generated.

Materials and Methods

Animals and sample collection. Crossbred gilts (Great Yorkshire x Dutch Landrace) which had shown 2 or more normal oestrous cycles (18-22 days) were used. The gilts were checked for oestrus with a vasectomized boar twice daily (09:00 and 15:00 h). The day of observing first standing oestrus was designated Day 0. Part of the gilts were artificially inseminated on Day 1, 24 h after the onset of standing oestrus. The gilts were slaughtered on Day 10 (N=8), 11 (N=6), 12 (N=7) and 13 (N=7) of cycle and Day 10 (N=9), 11 (N=11), 12 (N=9) and 13 (N=12) of pregnancy. The uterus was removed immediately after stunning and exsanguination. The horns of the pregnant gilts were flushed twice with 30 ml Dulbecco's phosphate-buffered saline (Gibco, Paisley, UK) and the recovered blastocysts were measured. Endometrium was dissected from the myometrium at the mesometrial side in the middle of the left uterine horn. The endometrial tissue was frozen in dry ice and stored until processing.

Receptor assay. All procedures were performed at 0-4°C. Endometrial tissue (500 mg) was minced in small pieces and pulverized with a microdismembrator (Braun, Melsungen, FRG). The tissue powder was extracted with 0.01 M phosphate buffer, (pH 7.5), containing EDTA (1 mM: Merck, Darmstadt, FRG). Cytosol was produced by centrifugation of the suspension for 1 h at 85000 *g*. Aliquots of the cytosolic fraction (250 µl) were incubated overnight with 100 µl of one of 6 dilutions of

[2,4,6,7-^{3}H]oestradiol (sp. act. 3.44 TBq/mmol: Amersham International, Amersham, UK), resulting in final concentrations of 0.3-2.0 nM. Competition was studied with 100-fold molar excess of non-radioactive ligand (DES: Sigma, St Louis, MO, USA) in parallel series of the two highest [^{3}H]oestradiol concentrations. Receptor-bound and unbound steroids were separated by centrifugation (10 min, 2000 *g*) after incubation for 10 min with a suspension of Dextran-coated charcoal (5% Norit A and 0.5% Dextran T-70: Pharmacia, Uppsala, Sweden) in buffer. An aliquot of the supernatant (750 µl) was added to 3 ml scintillation cocktail (Maxifluor: Baker, Deventer, The Netherlands) and radioactivity was determined by liquid scintillation counting (1900 CA Tri-Carb Liquid Scintillation Analyzer: Packard, Downers Grove, IL, USA). A control sample was used in every measurement to expose possible measurement errors; the inter-assay coefficient of variation was 11.3%. The concentration of cytosolic protein was measured according to the method described by Bradford (1976), using bovine serum albumine as a standard. The dissociation constant (Kd) and binding data were calculated by Scatchard plot analysis (Scatchard, 1949) after correction for non-specific binding according to Chamness & McGuire (1975). The cytosolic oestradiol receptor (ER_c) concentration was expressed as fmol/mg cytosol protein.

Statistical analysis. After ln transformation of the data differences in endometrial ER_c concentrations and Kd were tested for significance by analysis of variance and differences in endometrial ER_c concentration between gilts with spherical and gilts with filamentous blastocysts were tested for significance by Student's *t* test (SPPS Inc, 1988).

Results

Scatchard plot analyses indicated the presence of high-affinity cytosolic oestradiol receptors (ER_c) in endometrial

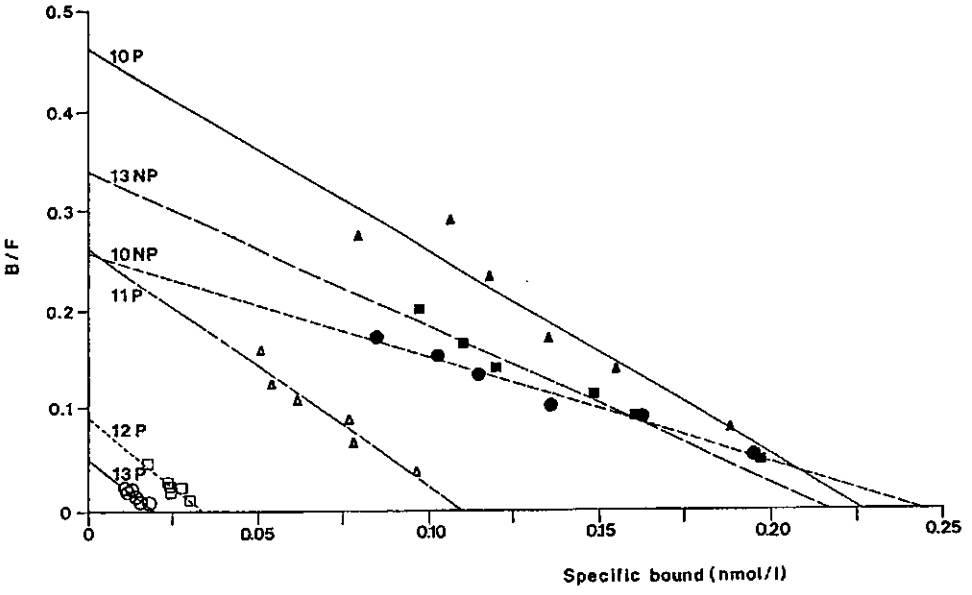

Fig. 1. Scatchard plot analysis of oestradiol receptors in endometrial cytosol from non-pregnant (NP) and pregnant (P) gilts collected on the day indicated beside each plot.

tissue of both non-pregnant and pregnant gilts on Days 10-13. Some Scatchard plots, representative for those obtained during Day 10-13 of cycle and pregnancy are shown in Fig 1.
The Kd of the endometrial ER_c was on average 0.40 nM (n=69, range 0.08-1.50 nM) and was not affected by day or reproductive state.

Endometrial ER_c concentration was significantly affected by day and reproductive state in cyclic gilts ($P<0.001$, Fig 2). In non-pregnant gilts endometrial ER_c concentration did not change from Day 10 to 13. During pregnancy endometrial ER_c concentration (y) declined during the consecutive days (x) ($y=187.3-13.7x$; $r=-0.53$; $P=0.0004$; Fig. 2). On Day 12 of pregnancy spherical blastocysts were recovered in 5 gilts, and filamentous blastocysts were recovered in 4 gilts. The

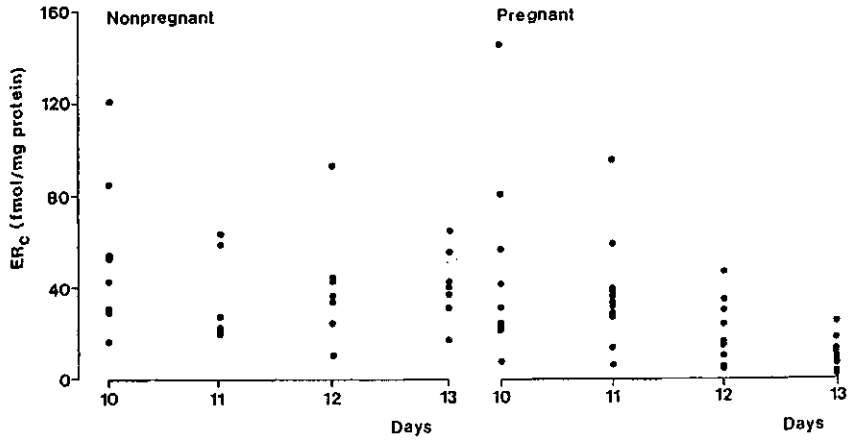

Fig. 2. Endometrial ER_c concentration on Days 10-13 in non-pregnant and pregnant gilts.

endometrial ER_c concentration was significantly larger ($P<0.01$) in gilts with spherical blastocysts (30.2 fmol/mg cytosol protein; n=5) than in gilts with filamentous blastocysts (9.8 fmol/mg cytosol protein; n=4).

Discussion

The high-affinity cytosolic oestradiol receptors with a Kd of on average 0.40 nM (ER_c) measured in endometrial tissue of both cyclic and pregnant gilts on Days 10-13, are comparable to those earlier reported for pig endometrium (Pack *et al.*, 1978; Deaver & Guthrie, 1980; Rexroad & Guthrie, 1984; Koziorowski *et al.*, 1984). In addition to these high-affinity oestradiol receptors also low-affinity high-capacity oestradiol binding sites may be present in endometrium of pigs (Tolton *et al.*, 1985).

In most studies endometrial ER_c concentrations in cyclic

gilts were higher in the luteal phase than in the early follicular phase (Deaver & Guthrie, 1980; Rexroad & Guthrie, 1984; Koziorowski *et al.*, 1984), although in 2 studies no differences were found (Pack *et al.*, 1978; Diekman & Anderson, 1979). In cyclic gilts around Days 10-13, endometrial ER_c concentrations have been reported on Day 12 which had not changed (Pack *et al.*, 1978), which were not significantly lower (Deaver & Guthrie, 1980) or significantly lower than on Day 10 (Rexroad & Guthrie, 1984). Part of these differences may be explained by the fact that in the studies of Deaver & Guthrie (1980) and Rexroad & Guthrie (1984) gilts of different breeds had been used. Since differences may exist in endometrial ER_c concentrations of cyclic gilts and cyclic sows of the same breed (Koziorowski *et al.*, 1984), differences between gilts of different breeds may also exist. In this study endometrial ER_c concentrations in cyclic gilts of the same breed measured on subsequent days, did not change from Day 10 to 13, which is in agreement with the data of Pack *et al.* (1978) for Days 10 and 12.

During pregnancy on Day 12 lower (Rexroad & Guthrie, 1984) or similar (Deaver & Guthrie, 1980) endometrial ER_c concentrations than compared with Day 10 have been reported. In both studies endometrial ER_c concentrations on Day 16 and 19 were lower than on Day 10 or 12 (Deaver & Guthrie, 1980; Rexroad & Guthrie; 1984). In this study endometrial ER_c concentrations declined significantly on Days 10-13 of pregnancy and were significantly affected by the developmental stage of the blastocysts.

At the time oestrogen synthesis by spherical blastocyst starts (Day 11; Fischer *et al.*, 1985; Mondschein *et al.*, 1985), endometrial ER_c concentration is high. While blastocysts develop into filamentous forms and oestrogen synthesis increases, endometrial ER_c concentration declines. Occupied nuclear ER concentration also seems to decrease during this period, since it is lower on Day 12 than on Day 10 (Rexroad & Guthrie, 1984) or even absent on Day 10 and Day 13 (Pack *et al.*, 1978, 1979).

Oestrogen interaction with genetic material in the nucleus has been shown to stimulate cytosolic oestrogen receptor resynthesis and synthesis of progesterone receptor (PR) (Döhler, 1987). Declining endometrial ER_c concentrations and cytosolic endometrial PR concentrations (J. van der Meulen, F.A. Helmond & C.P.J. Oudenaarden, preliminary data) from Day 10 to 13 suggest a down-regulation of ER_c concentration. As a consequence the action of oestrogens during Days 10-13 of pregnancy is probably a temporary effect of short duration. One of those effects is the oestrogen induction of calcium cycling across the endometrial epithelium (which in turn redirects the secretion of $PGF_{2\alpha}$ toward the uterine lumen, T.S. Gross & F.W. Bazer, unpublished data, quoted by Bazer *et al.*, 1989).

The results of this study demonstrate the existence of differences in ER_c concentrations between cyclic and pregnant gilts at the time blastocysts generate a first signal for the maternal recognition of pregnancy. The physiological significance of these differences, however, remains to be investigated.

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CHAPTER VIII

The effect of intrauterine oestradiol-17β administration on the cycle length of the non-pregnant pig

J. van der Meulen[1], F. Elsaesser[2], C.P.J. Oudenaarden[1] and F.A. Helmond[1]

[1]Department of Animal Physiology, Agricultural University, Haarweg 10, 6709 PJ Wageningen, The Netherlands;
[2]Institut fÜr Tierzucht und Tierverhalten, (FAL), Mariensee, 3057 Neustadt 1, FRG

submitted for publication

Summary

The effect of intrauterine administration of oestradiol-17ß (E2) on the life-span of the corpora lutea (CL) of non-pregnant gilts and gilts pregnant up to Day 10 was investigated. Injection of 380 ng E2 every 6 h from Day 11 (08:00 h) to Day 15 (02:00 h) into both uterine horns resulted in a cycle length of 21.7±1.0 days (mean ± s.d.) in non-pregnant gilts (N=7) and 22.4±1.7 days in gilts pregnant up to Day 10 (N=5). Implantation of 3 E2 releasing micropellets into each uterine horn of non-pregnant gilts on Day 10 of the cycle resulted in an interoestrous interval of 21.0±2.3 days (N=5). These results indicate that administration of a small, physiological or supraphysiological dose E2 into the uterus lumen (the presumed site of action in the maternal recognition of pregnancy), does not result in a substantial CL maintenance in non-pregnant gilts.

Introduction

The maternal recognition of pregnancy in the pig is supposed to be an oestrogen mediated mechanism (Bazer *et al.*, 1982). The oestrogens responsible for corpus luteum (CL) maintenance, are released by the blastocysts. *In vitro*, spherical Day 11-12 blastocysts of 5-7 mm, are able to convert labelled precursors into both oestrone (E1) and oestradiol-17ß (E2) (Perry *et al.*, 1976; Fischer *et al.*, 1985; Mondschein *et al.*, 1985). In uterine flushings of pregnant gilts, the total content of E2 and E1 increases between Days 10.5 and 14, but remains low in non-pregnant gilts (Geisert *et al.*, 1982a).

The anti-luteolytic effect of oestrogens can be demonstrated by systemic administration of large doses of oestrogens to non-pregnant gilts. Injection of oestradiol valerate (EV) or benzoate (EB)(Days 11-15; 5 mg/day) results in a reduced utero-ovarian vein (UOV) plasma $PGF_{2\alpha}$ concentration (Frank *et al.*, 1977) and an increased amount of $PGF_{2\alpha}$ and E2 in uterine

flushings (Geisert *et al.*, 1982b). The luteal life-span of these pigs is extended for a variable time period (146.5±74.8 and 92.0±11.2 days: Frank *et al.*, 1977, 1978; 41.0±4.4 days: Marengo *et al.*, 1986).

The injection of a smaller amount of E2 directly into the lumen of the uterus on Days 11-15 results in an increased UOV plasma progesterone concentration and a decreased UOV plasma $PGF_{2\alpha}$ concentration on Day 18 (Ford *et al.*, 1982), but causes only a three-day delay in return to oestrus (S.P. Ford & R.R. Magness unpublished results, quoted by Ford *et al.*, 1982). In the latter experiment E2 was injected only into one uterine horn.

In this study the effect of oestrogens on the life-span of the CL in the pig was reinvestigated. Plasma progesterone levels were measured and interoestrous intervals were recorded, after local administration of small amounts of E2 into both uterine horns of non-pregnant gilts and gilts pregnant up to Day 10, and after implantation of E2 releasing micropellets into both uterine horns of non-pregnant gilts.

Materials and methods

Animals and experimental design. Crossbred gilts (Great Yorkshire x Dutch Landrace) were checked for oestrus with a vasectomized boar twice daily (09:00 and 15:00 h). The length of the 2 cycles preceding the treatment cycle was recorded. About 5 days prior to the anticipated day of oestrus the gilts were fitted with an indwelling jugular vein catheter (PVC, 1.0 mm i.d., 1.5 mm o.d.). Blood samples were collected 3 times a day (09:00, 12:00 and 15:00 h). At the onset of standing oestrus (Day 0) 100 µg GnRH analogue (Ovalyse: Upjohn Company, Ede, The Netherlands) were injected i.m. and 5 gilts were artificially inseminated, 26 h after GnRH injection (Van der Meulen *et al.*, 1986).

In *experiment I*, in 14 non-pregnant and 5 pregnant gilts a catheter was inserted into the lumen of each uterine horn on

Day 10. In the pregnant gilts the blastocysts were flushed out of both uterine horns before inserting the catheters. After surgery the gilts received an injection every 6 h from Day 11 (08:00 h) to Day 15 (02:00 h) of either saline (non-pregnant, N=6) or E2 (Sigma, St Louis, MO, USA; 380 ng in saline) (non-pregnant, N=8; pregnant up to Day 10, N=5) into both uterine horns. The total volume of each intrauterine injection was 2.5 ml, supplemented with penicillin (100 IU/ml: Sigma) and streptomycin (400 µg/ml: Sigma).

In *experiment II*, at surgery on Day 10 three 1% E2 cylindric silastic micropellets (1 mm in length and 1 mm in diameter) were inserted into the lumen of each uterine horn in 5 non-pregnant gilts. The features of these micropellets have been recently described (Elsaesser *et al.*, 1989). In the horns of another 5 gilts, 3 empty pellets were implanted (control group). Before being placed into the uterine lumen, the micropellets had been incubated for 24 h in phosphate-buffered saline with 0.1% BSA at 37°C and the release of E2 was measured.

Blood sampling and checking for oestrus continued. After return to oestrus the gilts were slaughtered and the catheter position (*experiment I*) or the presence of micropellets (*experiment II*) was checked and the endometrial structure was examined. The recovered pellets were incubated again for 24 h in phosphate buffered saline with 0.1% BSA at 37°C to measure E2 release.

Surgery. All surgeries were carried out on Day 10 under general anaesthesia (metomidate-azaperone, Hypnodil-Stresnil: Janssen Pharmaceutica, Beerse, Belgium). The uterine horns were exposed by lateral laparotomy. In *experiment I*, a small incision was made just below the utero-tubal junction and a 60-cm section of a PVC catheter (1.0 mm i.d., 1.5 mm o.d.) was inserted into the lumen of the horn. The catheter occupied about 60-70% of the horn. This catheter was sealed at the tip and perforated at 10-cm intervals to allow uniform distribution of the injection fluid (Ford *et al.*, 1982;

Schneider *et al.*, 1983). Before suturing the incision an antibiotic was injected into the horn (Colicilline: 250 mg ampicilline and 25000 IU colistine: AUV, Cuyk, The Netherlands). The other 80-cm section of the catheter was used to exteriorize the catheter to the upper back of the gilt. The second horn was treated in the same way. Before the catheters were placed in the pregnant gilts, the blastocysts (average 10, range 5-15) were flushed out of both uterine horns (Van der Meulen *et al.*, 1988). In *experiment II*, the 3 micropellets were injected by a long needle (2 mm i.d., 2.5 mm o.d.) into each uterine lumen, one at about 15-cm below the utero-tubal junction, one at about 15-cm before the utero-cervical junction and one approximately in the middle of the horn.

Hormone analysis. All blood samples were collected into heparinized tubes, centrifuged and plasma was stored at -20°C until further analysis.

Plasma concentrations of progesterone were measured in all samples by RIA (Van der Meulen *et al.*, 1988). The intra-assay coefficient of variation was 6.5 % and the inter-assay coefficient of variation was 12.9%.

E2 release by the micropellets during incubation was measured after extraction and column chromatography by RIA, using a modification of the method described by Helmond *et al.* (1980). Samples of 100 µl were diluted in 900 µl phosphate buffer with 0.1% BSA and mixed with 1 ml acetate buffer (0.15 M, pH 4.1). The samples were extracted 3 times with diethyl ether (4 ml) after the addition of 1000 cpm of [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 evaporated under a stream of nitrogen, redissolved in 250 µl toluene:methanol (9:1, v/v) and applied to chromatography columns (8.0 x 0.7 cm) packed with Sephadex LH-20 (Pharmacia, Uppsala, Sweden; eluting solvent: toluene:methanol 9:1, v/v). The first 4.5 ml fraction was discarded and E2 was eluted in a final 3 ml. The E2 fraction was 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]E2 (87%). E2 concentrations were measured in duplo (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 oestrone (1.49%) and oestriol (0.21%). The antiserum was used in a working dilution of 1:25000. The sensitivity of the assay was 14 pg/ml at the 90% B/B_0 level. The intra-assay coefficient of variation was 16.5%. The amount of E2 is expressed in ng/ml/24 h after correction for procedural losses.

Table 1. Interoestrous intervals of 2 pretreatment cycles and the next cycle in which either saline or E2 was injected into each uterine horn.

Cycle	Cycle length (days) individual gilts	mean
	Non-pregnant gilts	
Pretreatment 1	22 22 20 20 20 21	20.8[b]
Pretreatment 2	19 20 20 19 19 19	19.3[a]
Injection saline	21 20 22 18 22 20	20.5
Pretreatment 1	22 21 19 21 21 20 21	20.7[b]
Pretreatment 2	20 18 20 21 18 20 19	19.4[a]
Injection E2	20 22 22 22 21 22 23	21.7[b]
	Gilts pregnant up to Day 10	
Pretreatment 1	18 21 22 22 20	20.6
Pretreatment 2	20 20 21 21 19	20.2[a]
Injection E2	23 21 21 22 25	22.4[b]

Values with different superscript are different ($P<0.05$).

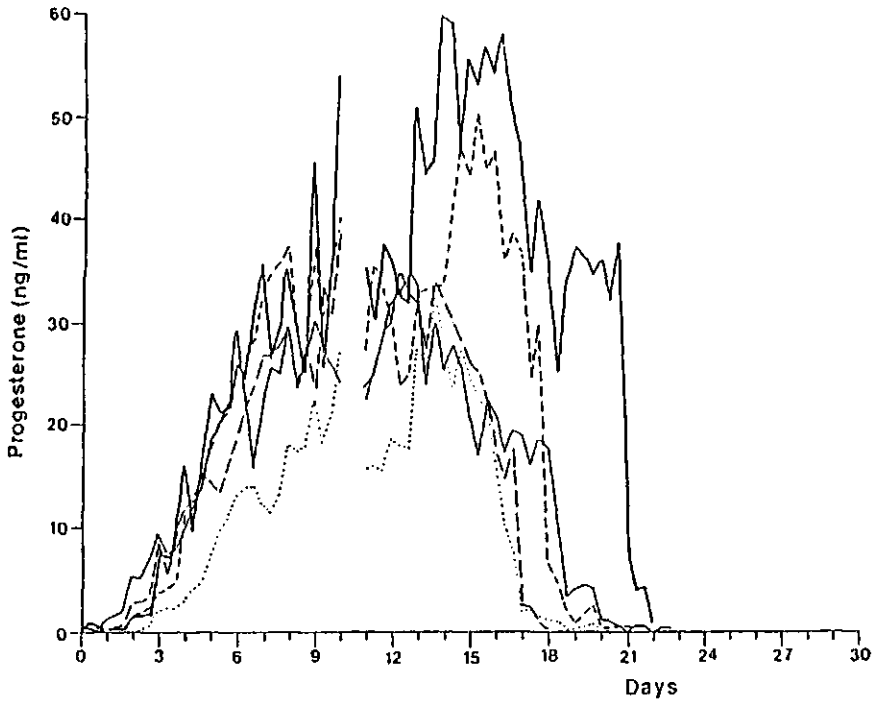

Fig. 1. The progesterone profile of 5 gilts pregnant up to Day 10 and injected with E2 into both uterine horns from Day 11-15.

Statistical analysis. Differences in length of pretreatment and treatment cycle were analysed by oneway analysis of variance (Snedecor & Cochran, 1980). All data are expressed as mean ± s.d.

Results

In *experiment I*, signs of uterine infection were observed at slaughter in 1 gilt after E2 injection, and the data of this gilt were excluded. The length of the 2 pretreatment cycles of

Table 2. Interoestrous intervals of 2 pretreatment cycles and the next cycle in which either 3 empty or 3 E2 pellets were inserted in each uterine horn.

Cycle	Cycle length (days) individual gilts	mean
Pretreatment 1	22 18 21 20 20	20.2
Pretreatment 2	22 20 20 19 20	20.2
Empty implants	21 19 21 20 21	20.4
Pretreatment 1	19 21 20 20 20	20.0
Pretreatment 2	20 20 19 20 19	19.6
E2 implants	20 25 20 21 19	21.0

the non-pregnant gilts differed significantly from each other ($P<0.05$; Table 1). The cycle length had not changed by injection of saline (20.5±1.5). The interoestrous interval after E2 injection had slightly increased in non-pregnant gilts (21.7±1.0 days) and gilts pregnant up to Day 10 (22.4±1.7 days). The maximum cycle length after E2 injection was 23 days in non-pregnant and 25 days in gilts pregnant up to Day 10 (Table 1). Compared with injection of saline, the time interval between luteolysis (progesterone ≤ 1 ng/ml) and return to oestrus had slightly increased in non-pregnant gilts (2.7±0.9 versus 1.7±1.2 days after saline injection), but had not changed in gilts pregnant up to Day 10 (1.8±0.4). In some gilts pregnant up to Day 10 a prolonged secretion of progesterone was observed (Fig. 1).

The E2 release by the micropellets during pre-incubation *in vitro* varied from 1700 to 5600 ng/pellet/24 h. On average 50% of the inserted micropellets were recovered after slaughter. Of the recovered pellets, two were floating in the uterine lumen and the others were imbedded in the endometrium. The

recovered E2 pellets still released E2 during *in vitro* incubation (40-73 ng/pellet/24 h). The cycle length of the gilts did not change by implantation of empty micropellets. Only in 1 gilt E2 implanted micropellets prolonged progesterone secretion and this gilt returned in oestrus on Day 25 (Table 2).

Discussion

The difference between the 2 pretreatment cycles in *experiment I* confirms that the cycle length may vary by about 2 days (Andersson & Einarsson, 1980). Therefore an increase in the interoestrous interval of at least 3 days is considered as an extension of the CL function. In this study, in the majority of the animals, no extension of the luteal phase was achieved by intrauterine injection of E2 or by the introduction of E2 releasing pellets in the uterus lumen.

Compared with the injection of 375 ng E2 4 times a day from Day 11 to Day 15 into one horn (S.P. Ford & R.R. Magness unpublished results, quoted by Ford *et al.*, 1982), injections into both horns in this study resulted almost in the same small average increase in cycle length (respectively 3 and 2.3 days). Geisert and Roberts (personal communication) also did not observe an extension of CL function after intrauterine E2 administration to non-pregnant gilts. Because E2 already plays a role before injections had started on Day 11 (e.g. morula-blastocyst transformation; Niemann & Elsaesser, 1987 and migration; Pope *et al.*, 1982), gilts pregnant up to Day 10 were also injected with E2, but a comparable small increase in cycle length was observed.

In vivo blastocysts release E2 probably more continuously. Implantation of E2 releasing micropellets evenly distributed over the uterus, may therefore be a better simulation of the *in vivo* situation. However, implantation of E2 releasing micropellets prolonged CL function in only 1 of 5 gilts. After implantation of 16 E2 releasing beads King & Rajamahendran

(1988) also did not find consistent and substantial prolonged cycles.

Provided that oestrogens released by the blastocysts are a major or the only factor for CL maintenance, both intrauterine E2 administrations should have been sufficient to induce an increase in cycle length of about 7-8 days (Geisert *et al.*, 1987; Van der Meulen *et al.*, 1988). In contrast to intrauterine oestradiol administration, i.m. injections of EV or EB do extend CL life-span for a variable time period. The progesterone secretion by luteal cells *in vitro* (Grazul *et al.*, 1986) and plasma progesterone concentrations (Guthrie & Rexroad, 1981; Ziecik *et al.*, 1986), however, show that EB injections do not mimic fully pregnancy. Injection of HCG on Day 12 also results in CL maintenance, without completely imitating pregnant plasma progesterone and $PGF_{2\alpha}$ concentrations (Guthrie & Rexroad, 1981; Guthrie & Bolt, 1983). It is possible that the mechanism by which large doses of i.m. administered oestrogen are luteotropic may affect the CL, either directly or via an extra-uterine organ (e.g. pituitary)(Flint *et al.*, 1983).

The results of both Ford *et al.* (1982) and this study indicate that administration of a small, physiological or supraphysiological dose of E2 in the uterus lumen, at the presumed site of action, does not result in a substantial CL maintenance in non-pregnant gilts. It is suggested that *in vivo* not only oestrogens but also other blastocyst secretory products (e.g. PGE_2; Akinlosotu *et al.*, 1986 or proteins) are necessary to bring about CL maintenance in the pig.

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Chapter IX

General discussion

Introduction

It is generally accepted that maternal recognition of pregnancy in the pig is an oestrogen mediated-mechanism (Bazer *et al.*, 1982), since Day-12 blastocysts convert labelled precursors into oestrogens during *in vitro* culture (Perry *et al.*, 1976) and i.m. injection of large doses of oestrogens on Days 11-15 (Bazer *et al.*, 1982) result in extension of CL function in cyclic gilts. On account of the fact that for continuation of pregnancy blastocysts must be present in both uterine horns between Days 10 and 12, and in one horn only after Day 12 (Dhindsa & Dziuk, 1968), it is generally assumed that maternal recognition of pregnancy occurs on Day 11 (Flint *et al.*, 1982).

Time of maternal recognition of pregnancy

To determine the time of maternal recognition of pregnancy, it seems important to reduce possible differences between gilts at the time of occurrence of the LH peak and subsequent ovulation and CL development with respect to the onset of standing oestrus (Helmond *et al.*, 1986). In order to synchronize the LH peak with the onset of standing oestrus, a GnRH analogue is injected at the time of observing first standing oestrus. Although it cannot be established whether this GnRH injection induces the preovulatory LH peak or enhances the endogenously occurring LH peak, LH peaks occurring more than 3 h after GnRH injection have never been recorded.

Comparison of progesterone levels of cyclic and pregnant gilts confirms that maternal recognition of pregnancy occurs before Day 14. Flushing of blastocysts out of both uterine horns on Days 10-13 shows that prolongation of CL life-span, depends particularly on the presence of blastocysts of a certain size, and not on the day of pregnancy. CL function is maintained in gilts in which at least some blastocysts were ≥8

mm in diameter. Blastocyst of this size are mostly present in gilts which are 11-12 days pregnant.

Since CL function is on average maintained for 7 days after flushing of the blastocysts on Days 12-13, a second signal seems necessary in order to maintain CL function for the whole period of pregnancy. As a result of i.m. injections of oestradiol valerate at different times, it has been suggested that such a second signal may occur on Days 14-16 and may also be an oestrogen-mediated mechanism (Geisert *et al.*, 1987). Failure of the second signal may account for the fact that 25.6% of all sows in commercial units return to service on Day 26.5±2.3 instead of Day 21 (Glossop & Foulkes, 1988).

Oestrogen synthesis by Day 11-13 blastocysts

CL maintenance for on average 7 days is achieved in gilts in which at least some large spherical blastocysts with a diameter of ≥8 mm were present. Determination of aromatase activity demonstrates the presence of oestrogen synthesis capacity already in small spherical blastocysts with a diameter of ≥2 mm. This indicates that a certain threshold release of oestrogen, as far as duration or absolute amount is concerned, has to be exceeded for CL maintenance. This threshold level may be achieved by the release of just a few blastocysts, since the cycle length is increased for on average 6 days in gilts with only 1-5 blastocysts (Polge *et al.*, 1966).

Around the time a first signal for CL maintenance is generated, both oestradiol-17ß and oestrone are released by the blastocysts during *in vitro* culture. These oestrogens are synthesized from endogenous precursors, and the release of oestradiol-17ß is larger than the release of oestrone. Also small amounts of conjugated oestrogens are released by the blastocysts.

A large variation in blastocyst size may occur at the time a first signal for CL maintenance is generated. Determination of

aromatase activity in spherical Day-11 blastocysts indicates that within gilts variation in blastocyst size is correlated with variation in oestrogen synthesis. *In vitro* culture of filamentous blastocysts indicates that variation in oestrogen synthesis is still present on Day 13, and also that filamentous blastocysts may differ in length (Bate & King, 1988).

Although variation in blastocyst size and oestrogen activity does not affect the first signal for maternal recognition of pregnancy, it may affect the generation of the second signal, necessary for CL maintenance for the whole duration of pregnancy. It has been suggested that the higher oestrogen synthesis of larger blastocysts on Day 11 may alter the uterine environment in such a way that their smaller littermates no longer can survive (Pope *et al.*, 1982). Advancing the uterine environment of pregnant gilts on Day 11 does not change the viability of the blastocysts on Days 12-14 (Gries *et al.*, 1989), but results in degenerating of conceptuses on Day 16 (Morgan *et al.*, 1987; Gries *et al.*, 1989). In these gilts a first signal for maternal recognition of pregnancy is generated, as indicated by elevated progesterone concentration on Day 20 (Gries *et al.*, 1989). At the time a second signal for maternal recognition of pregnancy is supposed to occur, however, a part of the conceptuses is degenerating. Whether a second signal is generated in gilts in which a part of the conceptuses is degenerating on Day 16 as a consequence of differences in oestrogen release on Day 11, may depend upon the number of remaining viable conceptuses. At least 6 conceptuses have to be present during early pregnancy to generate a second signal, since gilts with 1-5 blastocysts return to oestrus on Day 26.8±1.8 (Polge *et al.*, 1966).

Variation in blastocyst size on Day 11 may be related to differences in time of ovulation (Pope *et al.*, 1988). Injection of a GnRH analogue at the onset of standing oestrus, which has been used to synchronize the LH peaks between gilts, seems to decrease variation in blastocyst size on Day 11. This may have been a consequence of the sharper LH and FSH peak,

which may have reduced the duration of ovulation.

Intrauterine action of oestrogen

When oestrogen synthesis by spherical blastocysts starts, the endometrial cytosolic oestradiol receptor concentration is high, but it declines from Days 10 to 13 of pregnancy. The decline in endometrial cytosolic oestradiol receptor concentration suggests a down-regulation of oestradiol receptor concentration. Therefore, oestrogens exert during Days 10-13 of pregnancy probably a temporary effect of short duration.

In contrast to i.m. oestrogen injection, injection of oestradiol-17ß or implantation of oestradiol-17ß releasing micropellets into the uterine horns of cyclic gilts, does not change the interoestrous interval. I.m. injection of large doses oestrogens, however, may exert their effect on CL function either directly or via an extra-uterine organ, e.g. the pituitary (Flint *et al.*, 1983).

CL maintained by i.m. injected oestrogen differ from CL during pregnancy in steroid activity of luteal cells (Wiesak, 1989), progesterone profiles and CL life-span. I.m. injection of oestrogen increases prolactin concentration (Ziecik *et al.*, 1986) which may result in an increase in LH receptor level in the CL, and which synergizes with chorionic gonadotrophin to increase progesterone production by luteal cells *in vitro* (Flint *et al.*, 1983).

Prolactin also seems to be involved in the switch in direction of secretion of $PGF_{2\alpha}$ from an endocrine to an exocrine orientation (Mirando *et al.*, 1988). This switch has been suggested as the mechanism for CL maintenance in the pig (Bazer & Thatcher, 1977). In a perifusion device, endometrium from Day-14 cyclic gilts taken 6 or 12 h after oestradiol treatment responds to prolactin within 30 min by switching $PGF_{2\alpha}$ secretion from an endocrine to an exocrine direction. Prolactin does not affect the direction of $PGF_{2\alpha}$ secretion if

endometrium is taken before oestradiol treatment. (Mirando *et al.*, 1988). Prolactin also interacts with oestradiol enhancing uterine secretory activity (Young & Bazer, 1988). It is suggested that oestradiol induces endometrial receptors for prolactin which then allow prolactin to act on the endometrium to induce calcium cycling across the epithelium (Bazer *et al.*, 1989). Whether prolactin also plays a role *in vivo* remains to be investigated, since plasma prolactin levels are low during pregnancy (Ziecik *et al.*, 1986).
Proteins secreted by the blastocysts seem also to be involved in the switch in direction of $PGF_{2\alpha}$ secretion. *In vitro*, conceptus proteins enhance the effectiveness of oestrogen in redirecting the secretion of $PGF_{2\alpha}$ from the uterine vasculature (endocrine direction) toward the uterine lumen (exocrine direction)(Dubois & Bazer, 1988).

Conclusion

From this study it can be concluded that two signals are necessary for CL maintenance during the whole period of pregnancy. The first signal is generated in gilts in which at least some blastocysts are ≥8 mm in diameter. Already before the time of generating a first signal for CL maintenance, the blastocysts start to release both oestrone and oestradiol-17ß. These oestrogens exert an effect on the uterine endometrium, as can be indicated e.g. by changes in endometrial oestradiol receptor concentrations. However, in contrast to i.m. injection of large doses of oestrogen, intrauterine administration of small amounts oestradiol-17ß does not affect CL function. Blastocysts do not only secrete oestrogens but also proteins, prostaglandins and catecholoestrogens, and they may interact with oestrogens to affect CL function. Therefore, the first and probably also the second signal for maternal recognition of pregnancy do not seem to be simple oestrogen-mediated mechanisms.

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Summary

A vital link in a complex of physiological processes occurring during early pregnancy is the so-called maternal recognition of pregnancy: the prolongation of ovarian luteal function for continuation of progesterone secretion by an anti-luteolytic action of the developing embryos. Progesterone is crucial for the secretion of histotrophe, which in turn is essential for conceptus development and inhibition of uterine contraction activity. In the pig the corpora lutea (CL) remain the major source of progesterone throughout pregnancy.

To establish the time of maternal recognition of pregnancy in the pig (a) progesterone profiles of pregnant and cyclic gilts were compared (chapter III) and (b) blastocysts were flushed out of the uterine horns of gilts on Days 10-13 of pregnancy (chapter IV).

From Day 14, the progesterone concentration in pregnant gilts was higher than in cyclic gilts. This indicates that rescue of the CL during pregnancy occurs before Day 14.

Flushing of blastocysts out of both uterine horns on Day 10 of pregnancy resulted in a cycle with a normal progesterone profile and a normal length. Flushing of blastocysts on Days 11, 12 or 13 resulted in the maintenance of the CL for 3-13 days in those gilts in which at least some of the blastocysts were ≥8 mm or filamentous. Since CL function is only extended for on average 7 days, a second signal seems necessary to maintain the CL throughout pregnancy.

At the time that a first signal for maternal recognition of pregnancy is generated, the blastocysts start to secrete appreciable amounts of proteins, prostaglandins and oestrogens. So far, no anti-luteolytic role of proteins has been demonstrated in the pig, and data concerning an anti-luteolytic action of prostaglandin E_2 are contradictory. Oestrogens exert an anti-luteolytic effect as indicated by CL maintenance in cyclic gilts after i.m. injection of large doses of oestrogens.

The ability of blastocysts to synthesize oestrogens has been demonstrated by conversion of labelled precursors into both oestrone and oestradiol-17ß during *in vitro* culture. The conversion of androgens to oestrogens by aromatase activity has first been detected in litters with spherical Day-11 blastocysts.

To gain a clear understanding of the participation of each blastocyst in the initiation of oestrogen production, aromatase activity was determined in individual Day-11 blastocysts (chapter V). The oestrogen-synthesis capacity of blastocysts during the period that a first signal for maternal recognition of pregnancy is generated, was determined by measurement of oestrogen release by spherical and filamentous blastocysts during *in vitro* culture without supplementation of precursors (chapter VI).

Blastocysts flushed out of both uterine horns of 10 gilts on Day 11 of pregnancy varied in diameter and aromatase activity. The variation in aromatase activity reflected a difference between and within gilts. Of the total variation between all blastocysts, 33% was due to differences within gilts. A positive exponential relationship between blastocyst diameter and aromatase activity was determined, but this relationship differed between gilts.

Day-11 spherical blastocysts and clewed Day-13 filamentous blastocysts released both free and conjugated oestrone and oestradiol-17ß during 2 successive periods of 24 h *in vitro* culture. Individually collected and cultured Day-13 filamentous blastocysts developed trophospheres, and differences existed in the release of oestrone and oestradiol-17ß between and within gilts. Individual Day-13 filamentous blastocysts released on average 2.6 ng oestrone and 9.8 ng oestradiol-17ß per 24 h. In all cultures of spherical and filamentous blastocysts, the oestradiol-17ß release was significantly larger than the oestrone release. The data indicate that Day-11 and Day-13 blastocysts are able to synthesize oestrogens from endogenous precursors.

Assuming that CL function in cyclic gilts can be maintained by i.m. injection of large doses of oestrogens, several mechanisms have been proposed to explain the anti-luteolytic action of oestrogens in the pig. Oestrogens released by blastocysts, however, are supposed to exert their anti-luteolytic effect at the level of the uterine endometrial epithelium. Large doses of intramuscularly injected oestrogens, however, may act anti-luteolytically via a direct or indirect (pituitary) effect on the CL.

To investigate the influence of oestrogens at the level of the uterine endometrial epithelium, the concentration of endometrial oestradiol receptors at the expected time of maternal recognition of pregnancy in cyclic and pregnant gilts was determined (chapter VII).

Cytosolic oestradiol receptor concentrations did not change between Days 10-13 in cyclic gilts, but it decreased in pregnant gilts. The cytosolic oestradiol receptor concentration on Day 12 of pregnancy was higher in gilts with spherical blastocysts than in gilts with filamentous blastocysts.

To investigate the effect of a locally administered physiological dose of oestrogens, oestradiol-17ß was administered intrauterine in non-pregnant gilts and gilts pregnant up to Day 10 (chapter VIII).

Injection of 380 ng oestradiol-17ß every 6 h from Day 11 to Day 15 into both uterine horns resulted in normal cycles in non-pregnant gilts and gilts pregnant up to Day 10. Implantation of three oestradiol-17ß releasing micropellets into each uterine horn of non-pregnant gilts on Day 10 of the cycle resulted also in normal oestrous cycles. The results indicate that administration of oestradiol-17ß in physiological doses into the uterus lumen, the presumed site of action in the maternal recognition of pregnancy, does not result in CL maintenance in non-pregnant gilts.

From this study it may be concluded that a first signal for CL maintenance is generated in gilts in which at least some of

the blastocysts were ≥8 mm or filamentous, but a second signal is necessary to maintain CL function throughout pregnancy. Already before the time of generating a first signal for CL maintenance, the blastocysts start to synthesize both oestradiol-17ß and oestrone. The oestrogens exert an effect on the uterine endometrium, which is indicated by a change in endometrial oestradiol receptor concentration. However, since intrauterine oestradiol-17ß administration does not mimic the effect of the blastocysts, the first signal for maternal recognition of pregnancy is not a simple, merely oestrogen-mediated mechanism.

Samenvatting

Een belangrijke schakel in een complex van fysiologische processen die plaatsvinden tijdens de vroege dracht is de zogenoemde maternale herkenning van de dracht. Hieronder wordt verstaan het handhaven van de corpus luteum (CL) functie door middel van een anti-luteolytisch signaal. Deze anti-luteolytische werking wordt geïnduceerd door de zich ontwikkelende embryo's. Door het handhaven van de CL functie wordt de progesteronsecretie gecontinueerd. Progesteron is onmisbaar voor de secretie van histotroof, dat op zijn beurt essentieel is voor de ontwikkeling van de embryo's en voor de onderdrukking van de uteriene contractie-activiteit. In het varken blijven de CL tijdens de gehele dracht de belangrijkste bron voor de produktie van progesteron.

Om het tijdstip vast te stellen waarop de maternale herkenning van de dracht bij het varken plaatsvindt, zijn (a) de progesteronprofielen van cyclische en drachtige gelten vergeleken (hoofdstuk III) en (b) blastocysten uit de uterushoornen van gelten op Dag 10-13 van de dracht gespoeld (hoofdstuk IV).

Vanaf Dag 14 was de progesteronconcentratie in drachtige gelten hoger dan in cyclische gelten. Dit wijst erop dat de handhaving van de CL tijdens de dracht plaatsvindt voor Dag 14.

Het spoelen van blastocysten uit beide uterushoornen op Dag 10 van de dracht resulteerde in een cyclus met een normaal progesteronverloop en een normale lengte. Het uitspoelen van blastocysten op Dag 11, 12 of 13 resulteerde in het handhaven van de CL voor een periode van 3-13 dagen in die gelten, waarin tenminste enige van de uitgespoelde blastocysten groter dan 8 mm of filamenteus waren. Omdat de CL functie slechts verlengd wordt voor een periode van gemiddeld 7 dagen, lijkt een tweede signaal nodig om de CL in stand te houden voor de gehele duur van de dracht.

Op het moment dat een eerste signaal voor de maternale

herkenning van de dracht wordt afgegeven, starten de blastocysten met de produktie van aanzienlijke hoeveelheden proteïnen, prostaglandinen en oestrogenen. In het varken is tot nu toe niet aangetoond dat proteïnen een anti-luteolytische werking hebben, en met betrekking tot prostaglandine E_2 bestaan er tegenstrijdige gegevens over een anti-luteolytische werking. Oestrogenen oefenen een anti-luteolytisch effect uit, zoals blijkt uit handhaving van de CL in cyclische gelten na het intramusculair toedienen van grote doses oestrogenen.
Dat blastocysten in staat zijn tot synthese van oestrogenen, is aangetoond door middel van de omzetting van gelabelde precursors in oestron en in oestradiol-17ß tijdens *in vitro* kweek. De omzetting van androgenen in oestrogenen door middel van de zogenoemde aromatase-activiteit, is voor het eerst aangetoond in tomen met sferische blastocysten op Dag 11.

Om bij de aanvang van de oestrogeenproduktie inzicht te krijgen in de oestrogeensynthese van elke blastocyst, is de aromatase-activiteit bepaald in individuele Dag-11 blastocysten (hoofdstuk V). De oestrogeensynthesecapaciteit van blastocysten tijdens de periode dat een eerste signaal voor de maternale herkenning van de dracht wordt bewerkstelligd, is bepaald door het meten van de oestrogeenafgifte door sferische en filamenteuze blastocysten tijdens *in vitro* kweek in medium waaraan geen precursors waren toegevoegd (hoofdstuk VI).

Blastocysten die gespoeld waren uit de beide uterushoornen van 10 gelten op Dag 11 van de dracht, varieerden in diameter en in aromatase-activiteit. De variatie in aromatase-activiteit weerspiegelde een verschil binnen en tussen gelten. Van de totale variatie tussen alle blastocysten was 33% het gevolg van verschillen binnen gelten. Er bestond een positieve exponentiële relatie tussen de diameter van de blastocysten en de aromatase-activiteit, maar deze relatie verschilde tussen gelten.

Sferische Dag-11 blastocysten en kluwens Dag-13 filamenteuze blastocysten scheidden vrij en geconjugeerd oestron en

oestradiol-17ß uit tijdens twee opeenvolgende perioden van 24 uur in een *in vitro* kweek. Individueel verzamelde en gekweekte filamenteuze Dag-13 blastocysten ontwikkelden trophosferen, en er bestonden verschillen in oestron en oestradiol-17ß afgifte binnen en tussen gelten. Gemiddeld werd door filamenteuze Dag-13 blastocysten per 24 uur 2.6 ng oestron en 9.8 ng oestradiol-17ß afgegeven. In alle kweken van sferische en filamenteuze blastocysten werd significant meer oestradiol-17ß dan oestron afgegeven. De gegevens duiden erop dat blastocysten op Dag 11 en 13 oestrogeen produceren uit endogene precursors.

Uitgaande van het feit dat het functioneren van de CL in cyclische gelten gehandhaafd kan worden door intramusculair geïnjecteerde grote doses oestrogenen, zijn verschillende mechanismen geopperd om de anti-luteolytische werking van oestrogenen in het varken te verklaren. Oestrogenen die door de blastocysten worden afgegeven worden verondersteld hun anti-luteolytische werking uit te oefenen op het uteriene endometrium epitheel. Grote doses intramusculair geïnjecteerde oestrogenen, echter, zouden anti-luteolytisch kunnen werken via een direct of indirect (hypofyse) effect op de CL.

Om het effect van oestrogenen op het niveau van het uteriene endometrium te onderzoeken, is de concentratie van oestradiolreceptoren in het endometrium ten tijde van het verwachte tijdstip van de maternale herkenning van de dracht bepaald in cyclische en drachtige gelten (hoofdstuk VII).
Van Dag 10 tot 13 veranderde de oestradiolreceptorconcentratie niet in cyclische gelten, maar daalde in drachtige gelten. Op Dag 12 van de dracht was de oestradiolreceptorconcentratie in gelten met sferische blastocysten groter dan in gelten met filamenteuze blastocysten.

Om het effect van locaal toegediende fysiologische hoeveelheden oestrogenen te onderzoeken, is oestradiol-17ß intra-uterien toegediend aan cyclische gelten en gelten die tot Dag 10 drachtig waren (hoofdstuk VIII).

Injectie van 380 ng oestradiol-17ß elke 6 uur van Dag 11 tot

Dag 15 in beide uterushoornen van cyclische gelten en gelten drachtig tot aan Dag 10, resulteerde in normale cycli. Implantatie van drie oestradiol-17ß afgevende micropellets in beide uterushoornen van niet drachtige gelten op Dag 10 van de cyclus, resulteerde eveneens in normale oestrus cycli. Deze resultaten duiden erop dat toediening van fysiologische hoeveelheden oestradiol-17ß in het lumen van de uterus, niet leidt tot handhaven van de CL in niet drachtige gelten.

Uit dit onderzoek kan geconcludeerd worden dat een eerste signaal voor het handhaven van de CL wordt afgegeven in gelten waarin tenminste enige blastocysten groter dan 8 mm of filamenteus zijn, maar dat een tweede signaal nodig is om de CL in stand te houden voor de gehele duur van de dracht. Reeds voordat een eerste signaal voor het handhaven van de CL wordt afgegeven, beginnen de blastocysten met de productie van zowel oestron als oestradiol-17ß. De oestrogenen beïnvloeden het endometrium, zoals blijkt uit een verandering in de oestradiolreceptorconcentratie in het endometrium. Omdat intra-uterien toegediend oestradiol-17ß niet in staat is om het effect van de blastocysten na te bootsen, is het eerste signaal voor de maternale herkenning van de dracht echter niet een eenvoudig, enkel door oestrogenen geregeld mechanisme.

Curriculum vitae

Jan van der Meulen werd op 26 september 1960 te Leeuwarden geboren. Hij behaalde in 1978 het Atheneum-B diploma aan de Stedelijke Scholen Gemeenschap te Leeuwarden. In september van datzelfde jaar begon hij met zijn studie Biologie aan de Rijks Universiteit Groningen. In januari 1985 behaalde hij het doctoraal examen, met als hoofdvak gedragsfysiologie en als bijvakken voortplantingsfysiologie en ethologie. Op 1 februari 1985 trad hij als wetenschappelijk assistent in dienst van de Vakgroep Dierfysiologie van de Landbouwuniversiteit te Wageningen. Hij verrichtte gedurende drie en een half jaar het in dit proefschrift beschreven onderzoek. Bij dezelfde vakgroep (vanaf februari 1989 geheten Vakgroep Fysiologie van Mens en Dier) was hij vanaf 1 augustus 1988 tot 1 september 1989 werkzaam als universitair docent. Vanaf 1 september 1989 is hij als onderzoeker verteringsfysiologie werkzaam bij het Instituut voor Veevoedingsonderzoek (IVVO) te Lelystad.