Transformation of endometrium and fertility in late stages of pseudopregnancy in the rabbit

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Summary. Ovulation was induced in rabbits between Days 14 and 18 of pseudopregnancy by an intravenous injection of hCG. Induction of ovulation from Day 16 onwards led to normal progestational endometrial transformation. In rabbits injected on Day 14 or 15, a normal preimplantation endometrial morphology developed, but not earlier than 7 days after hCG (Day 14/15 + 7). Uteroglobin secretion was advanced during the second pseudopregnancy. After mating or artificial insemination, fertility was greatest on Day 18 of pseudopregnancy. Conception failed on Day 14 and embryo transfers were unsuccessful on Day 14 + 1. Transfers performed on Day 14 + 3, however, led to implantation and offspring, even though endometrial morphology did not correspond to the normal Day 3 preimplantational morphology at the time of transfer.

We conclude that (1) endometrial transformation typical of normal pseudopregnancy can be induced by ovulation during the regeneration phase of pseudopregnancy from Day 16 onwards; (2) fertilization and implantation can be achieved as early as Day 15 of pseudopregnancy; (3) an oestrous period with high mating activity and fertility occurs about 3 days later; and (4) Day 14 after hCG represents a limited time of functional change from pseudopregnancy to a fertile uterine cycle in the rabbit.

Introduction

In the pseudopregnant rabbit corpora lutea begin to regress about Day 12 after hCG (Hilliard, 1973). Luteal regression is indicated by decreasing concentrations of progesterone in blood serum (Harrington & Rothermel, 1977; Carlson & Gole, 1978; Ellinwood, Seidel & Niswender, 1979; Browning, Keyes & Wolf, 1980) and by typical morphological features of the endometrial epithelium (Arnold & Shorey, 1984; Busch, Winterhager & Fischer, 1986).

In the present study, induction of ovulation by a second hCG injection or by mating in later stages of pseudopregnancy, i.e. between Days 14 and 18 after hCG, and its effects on endometrial regeneration and on fertility have been investigated.

Materials and Methods

A total of 190 sexually mature female rabbits of mixed breed was used. The does were caged individually for at least 3 weeks in a controlled environment with 12 h light/24 h before induction of ovulation by 100 i.u. human chorionic gonadotrophin (hCG) i.v. (Prolan: Bayer, Leverkusen, FRG). The day of hCG injection was designated Day 0.

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Mating and artificial insemination (AI) studies. Artificial insemination (AI) was performed on Day 14, 15, 16, 17 or 18 according to Schlegel, Fischer, Beier & Schneider (1983). After 28 h the does were killed by a rapid intravenous injection of pentobarbitone sodium (75 mg/kg) (Nembutal: Abbott, CEVA, Bad Segeberg, FRG), the excised oviducts were flushed, and the eggs examined for 2- and 4-cell stages using a stereomicroscope (magnification $\times 20$ –40).

Part of the study, investigating mating behaviour and fertility, was performed as a field trial at the farm of our rabbit supplier in November, which is a poor breeding season for the rabbit. This time was chosen to minimize variation due to receptivity of the does. On Days 14, 16, 18, 20, 22 or 24, respectively, does (10 per day) were placed with bucks of proven fertility and the numbers of females mating and litters were recorded for each experimental day.

Embryo transfer experiments. About 3 h after recovery, embryos were transferred (5–8 per side) to recipients of the Day 14 series in which ovulation had been induced 1 or 3 days before transfer. Embryos were transferred into the oviducts (Day 1 embryos, 1 day after second ovulation) or into the uteri (Day 3 embryos, 3 days after second ovulation). Embryos were obtained from FSH-primed donors by flushing the oviducts and uteri, respectively, using Maurer's medium (Maurer, 1978) supplemented with 1.5% BSA. Transfers were performed by laparotomy through a mid-line incision and anaesthesia induced by 5 mg perphenazine/rabbit (Decentan: Merck, Darmstadt, FRG) i.m. and 40 mg thiobarbitone sodium/kg (Thiogenal: Merck) given i.v. Implantation sites were evaluated on Day 12 either by re-laparotomy or by killing the recipients.

Fig. 1. Experimental schedule for induction of ovulation in later stages of pseudopregnancy (≥ 3 rabbits/group for morphological studies, ≥ 2 for biochemical analysis). The experimental groups investigated were: Day 14 +1, +3, +5, +7; Day 15 +1, +3; Day 16 +1, +3; Day 17 +1, +3; Day 18 +1, +3, +5, +7.

Biochemical investigations. Uterine flushings were collected from rabbits, killed as described above, 1, 3, 5 or 7 days after the second hCG injection. The experimental groups are listed in Fig. 1. They are referred to as Day 14 +1, Day 14 +3, ..., Day 18 +7 (Fig. 1). The flushings were analysed for relative percentage of uteroglobin by disc gel electrophoresis (Beier, 1968, 1974, 1982; Maurer, 1971). Briefly, electrophoresis was performed in glass tubes using 7.5% polyacrylamide and a Tris–glycine buffer (pH 9.1). After staining with amido black and destaining the gel tubes were quantitatively evaluated by densitometry. The coefficients of variation (CV) were calculated by separating a randomly chosen sample 5 times in 3 different electrophoretic runs. The inter-assay CV was 28% and the intra-assay CV varied between 4 and 13%.

Fig. 2. Symplasm of uterine epithelium on Day 14 + 3. The multinucleated symplasm contains large secondary lysosomes with multilamellar sheets. $\times 7300$.

Fig. 3. Uterine epithelial cell (Day 14 + 3) exhibiting an apoptotic nucleus (arrow) as indicated by condensed chromatin at the margin of the nucleus. $\times 6000$.

Morphological investigations. On Days 14, 15, 16, 17 and 18 (all at +3 and +7), uteri of nulliparous does were fixed by perfusion and processed for freeze-fracture, transmission (TEM) or scanning electron microscopy (SEM) as described by Winterhager, Busch & Kühnel (1984).

Hormone assays. Peripheral blood samples were taken (3 rabbits/group) at Days 14–18 and Days 14–18 + 1, +3, +5, and +7, respectively. Plasma concentrations of progesterone were measured by radioimmunoassay ($[^3\text{H}]\text{PROG-RIA}$ 100: Institut National des Radioelements, Fleurus, Belgium) using an anti-rabbit progesterone antiserum. The samples were diluted 1:10 before measurement. The sensitivity of the assay is 7 ± 5.1 pg/assay tube. The intra-assay CV was 11% and the inter-assay CV was 15.6%. Plasma oestradiol- 17β was determined by ^{125}I -labelled oestradiol RIA ER 155 (Radio Isotopen Service, Würenlingen, Switzerland) with a sensitivity of 5 pg/ml, an intra-assay CV of 7% and an inter-assay CV of 9.6%. The oestradiol antiserum had been raised in rabbits.

Statistical analysis. Uteroglobin relative percentage values and hormone concentrations were subjected to statistical analyses by Duncan's multiple range test.

Results

Morphology of the endometrial epithelium

Epithelial morphology on Day 14 + 3 and Day 15 + 3 revealed striking differences compared to normal Day 3 endometrial transformation. Indications of cell destruction characterized the epithelial ultrastructure (Figs 2 & 3). Numerous, large symplasms which are normally not formed before Day 6 of pseudopregnancy (Winterhager *et al.*, 1984; Busch *et al.*, 1986) were still present (Figs 2 & 4)

Fig. 4. Scanning electron micrograph of a Day 14 + 3 uterine epithelium. Numerous symplasms can be recognized with intercalated groups of epithelial cells. $\times 200$.

Fig. 5. Uterine epithelium on Day 15 + 3 demonstrating that part of the epithelium consists of columnar epithelial cells with ovoid nuclei. $\times 4300$.

originating from the preceding cycle. These symplasms were obviously undergoing lysis, as indicated by large autophagic lysosomes in different stages of conversion and by cells with degenerating apoptotic nuclei (Figs 2 & 3). Sporadically, in Day 15 + 3 animals, small areas of columnar epithelium occurred such as are found in nonpregnant endometrium (Fig. 5).

In freeze-fracture replicas of Day 14 + 3 uteri, a broad tight junctional belt, comparable to those seen on Day 14 of pseudopregnancy (Busch *et al.*, 1986), was still present (Fig. 6). An epithelium

resembling that of the corresponding normal pseudopregnant stage was found not earlier than 7 days after hCG if hCG was given on Days 14/15 of pseudopregnancy. Typical morphological features for Day 7 of pseudopregnancy include dome-shaped cell protrusions, highly proliferated tight junctional belts (Fig. 7), and first signs of newly formed symplasms (Fig. 8). SEM overviews of the mucosal folds indicated that epithelial proliferation was sometimes slightly reduced in Days 14/15 + 7 uteri.

In contrast to Days 14/15, induction of ovulation as early as 1 day later, on Day 16 of pseudopregnancy, led to a normal endometrial transformation on Day 16 + 3. In normal pseudopregnant rabbits, i.e. with no second induction of ovulation, however, remnants of former symplasms and indications of cell destruction are still present on Day 16 (Busch *et al.*, 1986). Like those for Day 16, the Day 17 and 18 series showed a normal progestational endometrial transformation.

Uteroglobulin secretion

Compared to uteroglobulin concentration in normal pseudopregnant rabbits, ovulation during late stages of pseudopregnancy led to an earlier secretion of uteroglobulin 3 days after hCG (Fig. 9;

Fig. 6. Freeze-fracture replica of a uterine epithelial cell from a Day 14 + 3 animal. The lateral membrane exhibits a broad tight junctional belt similar to that of a Day 14 animal. The arrow-head indicates the direction of shadowing. am, apical membrane; lm, lateral membrane. $\times 22\,000$.

Fig. 7. Freeze-fracture replica of a uterine epithelial cell at Day 14 + 7. The tight junctional belt is greatly proliferated with strands running perpendicular to the uterine lumen. Such a proliferated tight junction is comparable to that of a Day 7 pregnant or pseudopregnant animal. The arrowhead indicates the direction of shadowing. am, apical membrane; lm, lateral membrane. $\times 27\,500$.

Fig. 8. Scanning electron micrograph of a Day 14 + 7 uterine epithelium. The typical dome-shaped protrusions of the cells are nearly free from microvilli (arrows), whereas the symplasms (big arrow) are densely covered with microvilli. $\times 330$.

Fig. 9. Uteroglobulin (as % of total protein) in uterine flushings from normal pseudopregnant rabbits (□) and from rabbits in which a second ovulation was induced between Days 14 and 18 of pseudopregnancy (▨). Data from all animals investigated (see Fig. 1) are pooled. Values are means $\pm$ s.e.m. from 7 (□) and 17 (▨) rabbits on Day 1; 2 and 10 on Day 3; 2 and 4 on Day 5; and 2 and 4 on Day 7, respectively.

$P < 0.01$). The early rise in uteroglobulin was confined to Day 3; no statistically significant differences were found on Days 1, 5 or 7 after the second hCG injection compared to normal pseudopregnancy. If, however, the various days of pseudopregnancy were considered separately, striking differences occurred between Day 14 and Days 15–18 (Fig. 10). In all animals of the Day 14 series (Days 14 + 1, + 3, + 5, + 7), lower uteroglobulin concentrations were found, particularly on Day 14 + 3 ($P < 0.01$). Advanced endometrial transformation, demonstrable morphologically as well as biochemically, therefore occurred in the rabbit when this transformation was induced in later stages of pseudopregnancy, but only if ovulation was induced later than Day 15.

Plasma progesterone and oestradiol

Plasma progesterone concentrations were still elevated on Days 14 and 15 ($P < 0.001$; Fig. 11), but decreased as early as 1 day after ovulation (Days 14–18 + 1; Fig. 12). The progesterone

concentrations did not fall to the basal values found on Days 16, 17 and 18 or in untreated non-pregnant controls (Fig. 11). Compared to rabbits in the Day 18 series, Day 14 does showed higher progesterone concentrations ($P < 0.05$) at 5 and 7 days after second ovulation. The low uteroglobin secretion on Day 14 + 3 (Fig. 10) was not reflected by lower progesterone concentrations.

Oestradiol concentrations in blood plasma were quite variable and seemed to be unaffected by progestational stage (Figs 11 & 12). On Day 14 of pseudopregnancy only, constantly but not statistically significant lower values were found ($P > 0.05$; Fig. 11). After hCG injection, higher concentrations, but again not statistically significant, were observed in all Day 14 groups (Days 14 + 1, + 3, + 5, + 7) compared to Days 16 and 18.

Fig. 10. Uteroglobin (as % of total protein) in uterine flushings from rabbits in which ovulation was induced between Days 14 and 18 of pseudopregnancy. Separate data for all groups investigated (see Fig. 1) are shown. Values are means $\pm$ s.e.m.; number of rabbits 5 for Days 14 + 1 and 15 + 1, and 2 for all other groups.

Fig. 11. Peripheral plasma progesterone and oestradiol concentrations in pseudopregnant rabbits. Control values were measured before treatment in nonpregnant, nonpseudopregnant rabbits. Values are means $\pm$ s.e.m. for 3 does/day of pseudopregnancy.

Fig. 12. Peripheral plasma progesterone and oestradiol concentrations in rabbits in which a second ovulation was induced between Days 14 and 18 of pseudopregnancy. Cumulative data of all animals investigated are shown at various days after the second hCG injection. Values are means $\pm$ s.e.m. for 9 does on Days 1 and 3 and for 6 does on Days 5 and 7 after second ovulation, respectively.

Fig. 13. Mating and littering rates in rabbits mated between Days 14 and 24 of pseudopregnancy (10 does/day of pseudopregnancy).

Fig. 14. Fertilization (examined 28 h after AI) and implantation rates (examined 12 days after AI) in rabbits artificially inseminated (AI) between Days 14 and 18 of pseudopregnancy. The numbers in the open columns represent the no. of eggs examined and the no. of does; those in stippled columns the no. of does examined.

Fertility

As shown in Fig. 13, most matings in late pseudopregnancy were recorded on Day 18 after hCG and resulted in the highest litter rates as well. Mating percentage and litter size decreased at later times. Although a few matings were observed earlier, no young were born to animals mated before Day 18. After artificial insemination, fertilization and implantation occurred 3 days earlier, i.e. at Day 15, but not on Day 14 of pseudopregnancy (Fig. 14). The best rates, however, were obtained on Days 17 and 18 after hCG.

To investigate the reasons for reproductive failure on Day 14 of pseudopregnancy and to discriminate between disturbances of fertilization and inability of the uterine milieu to support preimplantational embryonic development, we performed embryo transfers on Day 14 + 1 (75 embryos transferred into 6 recipients) and Day 14 + 3 (48 embryos transferred into 5 recipients). In contrast to transfers performed on Day 14 + 1 which did not lead to implantation, 38% of the transferred embryos did implant in 3 recipients when transferred 3 days after induction of ovulation on Day 14. From these pregnancies, normal living young were delivered by Caesarian operation on Day 29.

Discussion

The lifespan of corpora lutea in pseudopregnant rabbits has been reported to last about 17 days after hCG injection (Scott & Rennie, 1970). However, declining progesterone concentrations suggest that luteolysis starts earlier, about Day 12 (Harrington & Rothermel, 1977; Ellinwood *et al.*, 1979; Caillol, Dauphin-Villemant & Martinet, 1983), Day 13 (Browning *et al.*, 1980), or Day 14 after hCG (Carlson & Gole, 1978). Hoffman (1974) found morphologically intact corpora lutea on Day 13. Quatacker (1982) noticed the first histological signs of luteal regression at 12 days after hCG and these became pronounced on Day 16. Since endocrinological and morphological data are contradictory, we tried to identify the functional breakdown of pseudopregnancy in the rabbit.

Regarding termination of pseudopregnancy, various findings have to be kept in mind. As is known from light microscopical (Fischer, Winterhager, Busch & Beier, 1985) and electron microscopical investigations (Busch *et al.*, 1986), clear signs of epithelial regeneration are observed in the endometrium on Day 16 of pseudopregnancy. This observation correlates well with the decline in progesterone concentrations found in the present study (Fig. 11). The predominant uterine secretion protein during preimplantation in the rabbit, uteroglobin (Beier, 1967, 1968; Krishnan & Daniel, 1967), decreases after Day 10 (Johnson, 1972) or Day 12 (Fischer *et al.*, 1985). Mating behaviour and fertility (Fig. 13) indicate an oestrous period about Day 18 after hCG. This finding is confirmed by changes in sexual behaviour from unreceptive to a high rate of acceptance of males by pseudopregnant animals, as described previously by Caillol, Solari & Lefebvre (1984), and also by typical behaviour patterns such as nest-building activity and fur plucking which can be observed after unsuccessful matings about that time. We would therefore define the end of pseudopregnancy in the rabbit by functional criteria as being Day 18 after hCG. However, oestrus cannot be defined by morphological criteria because the uterine epithelium on Day 18 is still not restored (Busch *et al.*, 1986).

Regarding the period of epithelial restoration in late pseudopregnancy and its physiological consequences, three findings seem to be remarkable. Firstly, a normal pseudopregnant endometrial transformation as described previously (Beier & Kühnel, 1973, 1976; Davies & Hoffman, 1973, 1975; Suzuki & Tsutsumi, 1980, 1981; Busch, Kühnel & Mootz, 1981) can be achieved after induction of ovulation during this period, i.e. from Day 16 of pseudopregnancy onwards. Secondly, fertilization is found as early as Day 15, but not on Day 14, if the missing mating response is overcome by AI (Fig. 14). Thirdly, endometrial transformation allowing implantation can be initiated as early as Day 14 (see transfers on Day 14 + 3), but in this case the progestational endometrium shows clear

differences in morphology (Figs 2, 3, 4, 6) and secretory activity (Fig. 10) compared to normal preimplantational endometrium. In the Day 14 group, large symplasms and a broad tight junctional belt, normally most developed between Day 7 and Day 14 of pseudopregnancy (Fischer *et al.*, 1985; Busch *et al.*, 1986), are still present 3 days after the second hCG injection. Thin sections, however, reveal an enhanced transformation of symplasms by karyolysis. On Day 14 + 7, the normal Day 7 features of the uterine epithelium are restored, with newly formed symplasms and tight junctional strands which proliferate up to the basal portion of the lateral membrane (Winterhager & Kühnel, 1982; Winterhager *et al.*, 1984).

Enhanced luteal breakdown after ovulation on Day 14 is indicated by the steep decline in progesterone if concentration on Day 15 of normal pseudopregnancy (Fig. 11) is compared with the level on Day 14 + 1 of 2.2 ± 0.7 ng/ml. Finally, in contrast to the generally advanced secretion of uteroglobin 3 days after ovulation (Fig. 9), uteroglobin profiles stay low if ovulation is induced on Day 14 of pseudopregnancy (Fig. 10). The lower uteroglobin levels in the Day 14 animals may be explained by the large number of symplasms in uterine epithelium which may be less active in secretion. This would generally be in accord with findings of the inactivity of placental symplasms regarding protein synthesis (Kaufmann, 1983; Kaufmann, Nagl & Fuhrmann, 1983). Another reason could be an unrestored secretion phase as mentioned by Davies & Hoffman (1973, 1975).

An accelerated transformation of the endometrium occurred. On Days 16 and 18 after hCG, morphology is characterized by the persistence of degenerated cell remnants from the preceding cycle (Busch *et al.*, 1986). An appropriate ultrastructure, however, is restored within a few days after ovulation during this period. The accelerated transformation is sufficient to permit normal implantation as documented by the results after AI (Fig. 14) and embryo transfer, the latter even in animals of the Day 14 series (see Day 14 + 3 recipients).

Fertilization does not occur on Day 14 (Fig. 14) or 12 h later (no fertilization in 43 eggs from 5 does). On Day 15, however, 38% fertilized eggs (Fig. 14) and 20% does with implantation indicate that a limited period of functional change has appeared at this stage of pseudopregnancy. An insufficient milieu for embryonic development in the Day 14 group is indicated by the fact that embryos transferred on Day 14 + 1 did not implant. Failure of fertilization on Day 14 and of implantation on Day 14 + 1 may be caused by progesterone dominance before ovulation (Chang, 1950; Kennelly & Foote, 1965; Nutting & Mares, 1970; Chang, 1976; Vargas & Pauerstein, 1976). On Day 14 + 1, an advanced transport to a hostile uterus of the tubally transferred eggs might be involved (Adams, 1968; Glass, 1972; Takeda, Suzuki, Matsui, Terami & Tsutsumi, 1978). In an experiment comparable to our study, Adams (1968) noticed an accelerated tubal egg transport after ovulation induced in pregnant rabbits.

Transfers to the uterus performed on Day 14 + 3 led to embryo survival and implantation. Consideration of uterine morphology with widespread cell destruction at the time of transfer (Figs 2, 3, 4, 6) adds a new aspect to our previous understanding of the synchronization demands in this species (Chang, 1950; Hafez, 1963; Adams, 1974; Beier, 1974). In addition to successful post-partum breeding (Adams, 1967; Harned & Casida, 1969; Foxcroft & Hasnain, 1973), the Day 14 + 3 uterus provides another example where rabbit embryos can develop and implant in a milieu characterized by rapid endometrial regeneration. Synchronization between mother and embryo in the rabbit does not therefore require a morphologically properly proliferated 'synchronous' endometrium throughout preimplantation. The examination of features other than morphology may lead to a better understanding of the basic mechanisms for synchronization before implantation.

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