Plasma progesterone levels during pregnancy and pseudopregnancy in the hare (*Lepus europaeus syriacus*)

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Summary. A triphasic pattern of progesterone secretion was observed in female hares sampled throughout pregnancy and pseudopregnancy. After injection of hCG and artificial insemination (Day 1), progesterone values rose to a peak of 41.4 ng/ml about Day 14, remained at this level, then declined around Day 20 before increasing sharply to maximum levels of 67.7 ng/ml after midpregnancy (Day 28). Levels remained high for several days, then declined until Day 38, increased again until Day 41, before decreasing towards parturition. Progesterone levels were still high (37.5 ng/ml) 24 h before parturition. The progesterone pattern during pseudopregnancy closely resembled that observed during the first half of pregnancy: levels rose from Day 2 to a peak at Days 11–18, then declined sharply to baseline levels around Day 22. It is suggested that the control of progesterone secretion might be transferred from the pituitary to the placenta at the beginning of the second half of pregnancy.

Introduction

Hares (genus *Lepus*) are common mammals, with an almost world-wide distribution (Thenius, 1975), but because of the difficulty in breeding and handling these animals (Hediger, 1948; Puget, 1966; Olier & Montet, 1972) they have been little studied in the laboratory. Female hares show some degree of masculinization at birth (for references see Stavy, 1976), and we became interested in the endocrinological and behavioural aspects of pregnancy in the European hare (*Lepus europaeus*) in relation to fetal sexual differentiation of the young. The only information available on progesterone levels during pregnancy in the European hare is from a recent study on superfetation by Caillol & Martinet (1976); the results, based on 4 individuals, did not reveal a consistent pattern of progesterone secretion during pregnancy. In the present study the pattern of progesterone secretion throughout gestation and pseudopregnancy is described.

Materials and Methods

Animals

Female hares, *Lepus europaeus syriacus*, collected in the field as leverets at an average age of 5 days, were hand-reared and kept singly or in pairs in large outdoor pens with a floor area of 1 × 2 m (see Puget, 1970). Rabbit pellets, hay and water were always available; fresh carrots were given twice a week. A mineral supplement (Audevard, Limoges Cedex, France) was added to the water.

The hares were used in the present study when they were 9 months to 2 years old. Natural mating in hares usually requires the continual presence of a familiar male for every female, because introduction of an alien male results in vigorous aggression by the female and often injury of the male. Furthermore, the exact timing of conception in these circumstances is not known. To overcome both of these difficulties artificial insemination was used, permitting control over the timing and synchronization of pregnancies. Spermatozoa were obtained from the epididymides of a male anaesthetized with sodium pentobarbital (32 mg/kg: Diamond Laboratories, U.S.A.) and were diluted with physiological saline (0.15 M-NaCl) to a final volume of 5 ml. The diluted sperm suspension was stored

at 4°C and used within 1 h. Sperm motility was checked by microscopic observation before and after storage. The sperm suspension from each epididymis was sufficient to inseminate 5 females. Ovulation was induced by the intravenous injection of 50 i.u. hCG (Chorigon: Ikapharm, Ramat-Gan, Israel). Immediately after the hCG injection about 0.5 ml sperm suspension was introduced through a glass tube of 0.3 mm diameter directly into the vagina (for details see Stavy, Terkel & Marder, 1978). Pseudopregnant females were those which received an injection of 50 i.u. hCG but were inseminated with 0.5 ml 0.15 M-NaCl solution only. The day of insemination or sham insemination was considered Day 1 of pregnancy or pseudopregnancy respectively.

Ovariectomy

To determine whether the ovary is essential during the second half of pregnancy, 3 does were bilaterally ovariectomized on Day 29 of pregnancy and 3 other does were subjected to sham operation at the same time. Each doe was anaesthetized by intravenous injection of sodium pentobarbital (32 mg/kg) and the presence of viable fetuses was ascertained by laparotomy before ovariectomy.

Blood sampling

Two people were required for bleeding: one restrained the hare, sitting with the animal across his knees, while the second person obtained the blood. Blood was collected from the ear vein into a heparinized 5 ml syringe by using a 23-gauge needle. Blood samples were taken from 17 females throughout pregnancy and 6 females throughout pseudopregnancy; 3 of the females were bled during pregnancy and pseudopregnancy. The first blood sample was taken just before the hCG injection. Each pregnant animal was bled at 1- to 5-day intervals throughout gestation, usually between 10:00 and 13:00 h. The most frequent samples were obtained between Days 35 and 39, when the incidence of new pregnancies is highest. Pseudopregnant females were bled once every 5 days at the same time of day as the pregnant females. The blood was centrifuged (3000 g) at room temperature for 10 min and the plasma stored at -20°C until assayed.

Progesterone assay

Plasma progesterone concentrations were measured by the radioimmunoassay procedure described by Lindner & Bauminger (1974). The antiserum, a generous gift from Professor H. R. Lindner, Department of Hormone Research, Weizmann Institute of Science, was raised in rabbits to progesterone 11 α -hemisuccinyl-bovine serum albumin. For the determination of procedural losses, 1000 ct/min [1,2,6,7-³H]progesterone (sp. act. 94 Ci/mmol: Radiochemical Centre, Amersham, U.K.) were added to 0.5 ml plasma and shaken in glass-stoppered conical centrifuge tubes with 20 volumes petroleum ether (b.p. 40–60°C). The tubes were then immersed in a solid CO₂-acetone bath to freeze the aqueous phase and the organic phase was decanted into a second tube and dried under nitrogen. The dry eluate was redissolved in distilled acetone (1 or 2 ml). A portion of this solution (0.2 or 0.4 ml) was transferred to a counting vial, and the remainder was used for triplicate assay of progesterone concentration. Aliquots of 50–200 μ l of the redissolved extract were evaporated under nitrogen. Tris-buffer (200 μ l) containing 2.2% bovine γ -globulin (Fraction II, Sigma, St. Louis, Missouri, U.S.A.) was then added to the evaporated standards (progesterone was purchased from Ikapharm, Ramat-Gan, Israel) and samples. Antiserum to progesterone (300 μ l) was then added, and the tubes were incubated overnight at 4°C. Dextran-coated charcoal (200 μ l, 1% charcoal-0.1% Dextran T-70) was then used to separate the bound and free fractions. The specificity of the antibody in this system has been described by Kohen, Bauminger & Lindner (1975): cross-reaction with oestrogens, androgens and corticosteroids was <0.1%, there was a slight cross-reaction with 17 α -hydroxyprogesterone (2%) and the 11-oxygenated derivatives of progesterone (44% for the 11 α -hydroxy- and 8% for 11 β -hydroxyprogesterone).

The lower limit of sensitivity of the assay, calculated as the hormone concentration which corresponded to the lower 95% confidence limit of the non-hormone containing tubes within each assay, averaged 15 pg progesterone/tube. Intra-assay precision was examined by measuring the progesterone concentration of replicates from a pool of luteal-phase plasma samples of normal women. The mean $\pm$ s.d. progesterone concentration from 10 samples was 14.25 $\pm$ 0.45 ng/ml.

Inter-assay precision was examined by analysis of progesterone concentration from a pool of female serum run on separate days in each assay: in 6 runs, the mean progesterone concentration was 8.12 ± 0.23 (s.d.) ng/ml. For estimation of blank values, buffer (0.5 ml) was extracted with petroleum ether (20 volumes) and subjected to the radioimmunoassay procedure as described above. The mean progesterone concentration found in the 0.5 ml samples of petroleum ether-extracted buffer examined on separate runs was 10 ± 7 pg.

Results

The progesterone concentrations before insemination were <1 ng/ml. The results presented in Text-fig. 1 suggest a triphasic pattern of progesterone secretion during pregnancy. In Phase 1, progesterone concentration increased gradually from Days 2–3 and reached a maximum (41.4 ng/ml) at about Day 14 of pregnancy. These levels were maintained during the 3rd week of pregnancy. Phase 2 began in the 4th week with a rapid rise of progesterone levels, reaching a maximum level (67.7 ng/ml) around Day 28. Progesterone levels then declined to a mean of 37.5 ng/ml about 1 week before parturition. The maximum levels of progesterone in Phase 2 were about 60% higher than those observed at the peak of Phase 1. In Phase 3, progesterone levels rose again to a third peak (55 ng/ml) a few days before parturition. Relatively high progesterone levels (37.5 ng/ml) were still observed 24 h before parturition, but on the day after parturition, progesterone had again reached baseline levels (<1 ng/ml).

Text-fig. 1. Mean $\pm$ s.e.m. concentrations of progesterone in the peripheral plasma of hares throughout pregnancy. Samples taken at 2-day intervals are combined and plotted on even-numbered days, except for samples on Days 35 to 39 which are plotted at daily intervals. The numbers of hares at each point are indicated. AI = artificial insemination; P = parturition.

The general pattern of progesterone secretion during pseudopregnancy resembled that of Phase 1 secretion during pregnancy. Progesterone rose gradually after the induction of pseudopregnancy, reaching a maximum between Days 11 and 18, then declining to baseline levels between Days 20 and 24. In each of the 3 females in which progesterone levels were measured during pregnancy and

pseudopregnancy, the duration, pattern and amplitude of the hormone levels during Phase 1 of pregnancy were strikingly similar to those observed during her own pseudopregnancy (Text-fig. 2). In each individual, the decline of progesterone to baseline levels at the termination of pseudopregnancy coincided with the progesterone decline at the end of Phase 1.

Text-fig. 2. Plasma progesterone concentrations in a single hare (No. 37) illustrating the hormone profile during pregnancy (●) and pseudopregnancy (○). AI = artificial insemination; P = parturition.

The 3 females which were ovariectomized on the 29th day of pregnancy aborted within 36 h. Progesterone levels at this time were less than 1 ng/ml. Two of the sham-operated females gave birth normally, while the third aborted 5 days after the operation. We attribute the abortion of this animal to severe stress, because the abortion occurred shortly after the first bleeding and the animal seemed nervous and upset.

Discussion

In spite of the individual differences in progesterone levels and the variable interval between blood samples, a common pattern of plasma progesterone levels during pregnancy was observed in all the hares sampled. The pattern is, however, different from that in the rabbit, which is taxonomically the closest living relative of the hare. In the rabbit, progesterone levels rise gradually from the 3rd day of pregnancy, reach peak levels around midpregnancy, and decline gradually toward the end of pregnancy (Mikhail, Noall & Allen, 1961; Hilliard, Spies & Sawyer, 1968; Challis, Davies & Ryan, 1973; Hilliard, Scaramuzzi, Penardi & Sawyer, 1973). We observed that the pattern of progesterone secretion in the hare resembles that of the rat and mouse. In the latter two species, the pituitary can be removed at any time after midpregnancy without interrupting pregnancy. However, in the rabbit the pituitary is essential throughout pregnancy, hypophysectomy at any stage causing abortion through failure of the corpora lutea (Robson, 1937, 1940; Westman & Jakobsohn, 1937; Spies, Hilliard & Sawyer, 1968).

Three lines of evidence suggest that during pregnancy the control of progesterone in the hare is transferred from the pituitary to the placenta at the end of Phase 1 of secretion. (1) The progesterone profile observed during pregnancy in the hare is similar to that found in the rat (Pepe & Rothchild, 1972; Morishige, Pepe & Rothchild, 1973) and the mouse (Murr, Stabenfeldt, Bradford & Geschwind, 1974; Virgo & Bellward, 1974). The decline of progesterone levels at the end of the first half of pregnancy in these rodents has been shown to be due to the cessation of pituitary control of progesterone secretion, while the placenta has not yet achieved complete steroidogenic control (for a discussion see Murr *et al.*, 1974). (2) The termination of pseudopregnancy in the hare occurs at the

same time as the end of Phase 1 of progesterone secretion (see Text-fig. 2). It has been shown both in the rat (Pepe & Rothchild, 1974) and the mouse (Choudary & Greenwald, 1969; Murr *et al.*, 1974) that the termination of pseudopregnancy coincides with the time at which the pituitary ceases to be the main luteotrophic source and progesterone therefore declines rapidly to basal levels. (3) In the hare, ovariectomy in the second half of pregnancy resulted in immediate abortion, indicating that the ovary remains the main source of progesterone throughout pregnancy (for review see Hilliard, 1973; Ryan, 1973) as in other mammals which abort under the same conditions (rat, rabbit). Thus placental progesterone is either inadequate or absent. It is believed that in the above two species, which cannot maintain pregnancy following ovariectomy, the rise in progesterone during the second half of pregnancy is due to the transfer of control of progesterone secretion from the pituitary to the placenta (as discussed by Murr *et al.*, 1974). Perhaps this is also the case in the hare.

The phenomenon of superfetation is common in the European hare: the animals can mate and conceive up to 7 days before parturition (Hediger, 1948; Martinet, Legouis & Moret, 1970). In most mammals, high progesterone levels prevent ovulation by blocking LH secretion or by a direct inhibition of the ovaries (Schwartz & Talley, 1968; Van Rees, Van Dielen, Bijleveld & Muller, 1968; Beyer & McDonald, 1973; Labhsetwar, 1975). In many species, exogenous progesterone blocks sexual behaviour during pregnancy; in the rabbit matings do occur during the last few days of pregnancy but the progesterone levels are very low (Beyer & Rivaud, 1969; Beyer, Vidal & McDonald, 1969). Because our hares were housed individually, no mating behaviour before parturition was observed. Nevertheless, the drop in progesterone levels just before parturition occurred at the time when the incidence of new pregnancies is highest (Martinet *et al.*, 1970; Caillol & Martinet, 1976).

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