Effect of ovariectomy at two periods of the year on LH and FSH basal concentrations and pituitary response to LHRH in the brown hare (*Lepus europaeus*)

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Summary. In the brown hare, fertile mating takes place from the beginning of December to September. Seasonal variations of basal concentrations of LH and FSH, and pituitary response to a monthly i.v. injection of LHRH were studied in intact control females and in females ovariectomized during the seasonal anoestrus (OVX1) or during the breeding season (OVX2). In intact females, both basal and LHRH-stimulated LH levels showed an annual variation, with minimal values during anoestrus. During the breeding season, the LH response to LHRH exhibited a biphasic pattern. In contrast, there was no clear seasonal variation in basal and LHRH-stimulated FSH concentrations. After ovariectomy during anoestrus, basal LH remained low for 2 months and began to increase in December. After ovariectomy during the breeding season, LH basal concentrations increased within a few days after the operation. Thereafter, LH values remained high in both groups of females until September, and decreased significantly as in intact females. The pattern of LH release after LHRH remained monophasic in the two groups of ovariectomized females. In OVX1 females, the LH response increased as early as October, was maximum from December to April and decreased progressively until October. In OVX2 females, the LH response decreased regularly after ovariectomy to a minimum in October. In the 2 groups of ovariectomized females, basal FSH concentrations and pituitary response to LHRH rose rapidly after ovariectomy and did not vary significantly thereafter. These results showed a direct central effect of season on the regulation of basal concentrations of LH, modulated by a negative feed-back of ovarian secretions during the breeding season. In intact hares, the enhanced LH response after LHRH during the breeding season was related to an acute positive effect of ovarian secretions. The regulation of FSH was less dependent on season and remained under a negative control of the ovary throughout the year.

Keywords: brown hare; LH; FSH; LHRH; ovariectomy; season

Introduction

The brown hare, *Lepus europaeus*, is a seasonal polyoestrous induced ovulator: under natural conditions of daylength and temperature, the first fertile matings occur at the beginning of December, and the first parturitions take place at mid-January. The last matings occur at the beginning of September. Photoperiod is one of the factors regulating the length and timing of the breeding season (Martinet & Moret, 1971; Meunier & Martinet, 1986). In this species, the annual reproductive cycle is related to changes in LH basal concentrations and in the pituitary response to LHRH: both values increase from January to a maximum in July, and decrease in August to a

nadir from September to December. From January to August, the pattern of LH release induced by an LHRH injection is modified and a 2nd peak of LH appears 2 h after injection (Caillol *et al.*, 1986). Similar seasonal fluctuations of LH concentrations have been described by Davis & Meyer (1973) for the snowshoe hare (*Lepus americanus*). To our knowledge, no data are available concerning seasonal variations of circulating FSH concentrations in the brown hare. The only information relates to the pituitary FSH content in the snowshoe hare in which minimal values in November–December are followed by an increase from March to a maximum in June (Davis & Meyer, 1973).

In the first part of this paper, we have studied the seasonal variations of FSH (basal values and response to an exogenous LHRH stimulus). In the second part, we have determined the relative roles of ovarian secretions and of environmental cues on the regulation of both basal and LHRH-stimulated concentrations of LH and FSH.

Materials and Methods

Animals. Hares were born in captivity and kept isolated outdoors at weaning (1 month). The control and experimental females were 6 months old at the beginning of the experiment. Two groups of 6 females receiving alternately every 2 months an i.v. injection of 5 µg LHRH (1.7 µg/kg body weight; Stimu-LH: Roussel, Paris, France) were used as control. The basal and stimulated LH and FSH concentrations did not differ in these 2 groups of females and so the same symbol has been used in the figures. Two groups of 5 experimental females were ovariectomized under pentothal anaesthesia either during the seasonal anoestrus (19 September, OVX1) or during the breeding season (27 March, OVX2). They received an i.v. injection of 5 µg LHRH before ovariectomy and once a month after ovariectomy.

Blood was obtained from the ear veins of unanaesthetized animals before LHRH injection and 15, 30, 60, 90, 120, 300 min and 24 h after injection. To study basal concentrations of LH and FSH, blood was sampled every 2 weeks around ovariectomy in experimental females. Serum samples were prepared after centrifugation at 4°C (5000 g) and stored frozen until assayed.

Measurement of LH. LH was measured as previously described (Mondain-Monval *et al.*, 1985). The rabbit anti-ovine LH antiserum was kindly supplied by Dr G. D. Niswender (GDN 15); the highly purified sheep LH (LER 1056-C2) used as a tracer and the dog LH (LER 1685-1) used as a standard were provided by Dr L. E. Reichert. The sensitivity of the LH assay was 0.4 ng/ml, and intra- and inter-assay coefficients of variation were 4 and 6%, respectively.

Measurement of FSH. Hare FSH was measured using a heterologous double-antibody radioimmunoassay as described previously for the fox (Mondain-Monval *et al.*, 1988). The rabbit anti-human FSH antiserum (M 94) (McNeilly *et al.*, 1976) was used with highly purified sheep FSH (LER-1976 A2) as tracer, and with a highly purified dog FSH preparation (LER-1685-3A) as standard, supplied by Dr L. E. Reichert.

The antiserum was characterized by McNeilly *et al.* (1976) and cross-reacts with FSH from several mammalian species. Parallelism in the displacement curves caused by dog FSH, sheep FSH and dilutions of hare pituitary extract or serum samples from ovariectomized hares was tested. The accuracy of the assay was determined by the measurement of known amounts of dog FSH or diluted hare pituitary extracts added to serum containing low levels of FSH. The detection limit was defined as the minimum concentration of dog FSH capable of causing a significant displacement ($P < 0.05$) of radiolabelled FSH from the antiserum. The precision was assessed by the repeated measurement of 2 pools of serum.

Because of a lack of a purified hare FSH, a highly purified dog FSH preparation was used as standard. The FSH values are expressed as ng LER-1685-3A equivalents/ml. Samples and reference preparations were measured in duplicate.

Statistical analysis. All concentrations of LH and FSH are expressed as mean $\pm$ s.e.m. Data for measurements taken over time were compared by Student's *t* test for paired dependent values for 2 successive samples. Data for non-repetitive variables were analysed by analysis of variance. Differences between groups were assessed for significance by the LSD test.

Results

Validation of the FSH assay

Specificity. The method proved to be specific for hare FSH. The binding of ^{125}I -labelled FSH to antiserum M 94 was displaced in a parallel manner by hare crude pituitary homogenates and purified dog FSH (Fig. 1). Serial dilutions of serum from ovariectomized doe hares were parallel to

the inhibition curves obtained with hare pituitary extract and dog FSH (Fig. 1). Furthermore, high concentrations of LH in serum from pregnant hares injected with LHRH did not affect FSH values which remained undetectable. In contrast, all the hares showed an increase in FSH concentrations after ovariectomy (see below, Fig. 3).

Fig. 1. Inhibition curves for dog FSH (LER 1685-3A) prepared in assay buffer with (▲—▲) or without (□—□) hare serum and dose-response curves for dilutions of pituitary extract (○—○) and for dilutions of serum from ovariectomized hares (●—●).

Accuracy. The recoveries of known amounts of dog FSH (16–2048 ng) added to serum from hares during anoestrus and previously measured at <20 ng/ml were $96.8 \pm 5.0\%$.

Sensitivity and precision. Under routine conditions the assay was sensitive to 20–30 ng/ml serum. The proportion of total radioactivity bound by the antiserum in the absence of hormone ranged from 15 to 35%. The intra- and inter-assay coefficients of variation were 8.0 and 10.6%, respectively.

Annual variations of basal concentrations of LH and FSH in intact and ovariectomized females

LH. In intact females, LH values remained undetectable during seasonal anoestrus, and rose slightly from March to August (Fig. 2).

In females ovariectomized during the seasonal anoestrus (OVX1), the basal concentration of LH began to increase significantly 3 months after surgery ($P < 0.05$), in December, remained around 10 ng/ml during the breeding season, and decreased in September, during the anoestrus

Fig. 2. Seasonal variations in basal concentrations of LH in 6 control (●—●), 5 OVX 1 (○—○) and 5 OVX 2 (▲—▲) females. Values are mean $\pm$ s.e.m. The arrows indicate the time of ovariectomy. In the insert, mean $\pm$ s.e.m. LH levels in OVX 1 (○—○) and OVX 2 (▲—▲) females are plotted from the day of ovariectomy (= Day 0).

(Fig. 2). When ovariectomy was performed during the breeding season (OVX2), the concentration of LH rose significantly as soon as 8 days after ovariectomy ($P < 0.05$), remained around 5 ng/ml from April to September, and returned to low values during anoestrus ($P < 0.05$). Between April and September, the values of LH were significantly lower in OVX2 than in OVX1 groups ($P < 0.05$).

FSH. In intact females, FSH basal concentrations fluctuated between 20 and 40 ng/ml, without any significant seasonal variation ($P > 0.05$) (Fig. 3).

Ovariectomy performed during the seasonal anoestrus was followed by an immediate rise in FSH concentrations. At 2 weeks after surgery, the mean FSH level was 188 ± 34 ng/ml ($P < 0.01$). Maximum FSH values were reached 1 month later and remained around 400 ng/ml until the end of the experiment (Fig. 3). Ovariectomy performed during the breeding season was also followed by a very rapid and transient rise in FSH concentrations, up to 452 ± 85 ng/ml 8 days after the operation ($P < 0.01$). Thereafter, these levels decreased significantly by 100%, and remained at a plateau significantly lower than in the previous group ($P < 0.05$).

Annual variations of the pituitary response to LHRH injection

FSH and LH profiles after LHRH injection at different periods of the year. Whatever the period of the year, in intact and ovariectomized females, LHRH injection was followed by a release of LH and FSH (Fig. 4). In intact females, during the seasonal anoestrus, the release of LH reached a maximum 15 min after injection and returned to basal value 2 h later. During the breeding season, a second peak of LH occurred 2 h after the injection. The release of FSH occurred 15 min after LHRH and lasted about 5 h, without any significant seasonal variation in the pattern of release in the 5 h following LHRH. Nevertheless, during the breeding season, FSH concentrations were significantly enhanced 24 h after LHRH injection, when compared to the values observed during

Fig. 3. Seasonal variations in basal concentrations of FSH in 6 control (●—●), 5 OVX 1 (○—○) and 5 OVX 2 (▲—▲) females. Values are mean $\pm$ s.e.m. The arrows indicate the time of ovariectomy. In the insert, mean $\pm$ s.e.m. FSH levels in OVX 1 (○—○) and OVX 2 (▲—▲) females are plotted from the day of ovariectomy (= Day 0). ** $P < 0.01$.

the seasonal anoestrus (February to July: 103 ± 18 ng/ml, $n = 23$, vs September to December: 25 ± 4 ng/ml, $n = 18$, $P < 0.05$).

In females ovariectomized during the seasonal anoestrus (OVX1), the peak level of LH occurred 15 min after LHRH, and the basal value was reached 2 h later. After ovariectomy during the breeding season (OVX2), the same profile was found: the 2nd LH peak found during the breeding season in intact hares never appeared in ovariectomized females.

In the two groups of experimental animals, the amount of FSH released during 5 h after LHRH was significantly higher following ovariectomy ($P < 0.01$). There was no significant seasonal variation in the FSH profile after LHRH, and, in contrast to the control group during the breeding season, there was no increase in FSH concentrations 24 h after LHRH. Furthermore, the pituitary response remained lower in OVX2 than in OVX1 females (Fig. 4).

Seasonal changes in LH released 15 min after LHRH. In intact females, the peak level of LH reached 15 min after LHRH was minimum from September to December, increased between January and August, and returned to low values in September (Fig. 5a). In the OVX1 group, the peak level increased significantly as early as October ($P < 0.05$), was maximum from December to April, and then decreased progressively until October. In the OVX2 group, the peak values fluctuated between 30 and 50 ng/ml, without any significant seasonal variation (Fig. 5a). When compared to the control animals, the values in OVX1 females were significantly higher from October to December, and significantly lower from June to August. The values in the OVX2 group decreased slightly after the operation, and became lower than those of the control group until September and lower than in the OVX1 group until May ($P < 0.05$).

Seasonal changes in LH released 120 min after LHRH. In intact females, the concentration of LH reached 2 h after LHRH was minimum until December, began to rise in January to a maximum between March and July, and returned to low values in September. When experimental females were compared to the control, the LH values were significantly lower from March to July (OVX1), or immediately after ovariectomy, from April to July (OVX2) (Fig. 5b).

Fig. 4. Effect of LHRH injection (5 µg i.v.) at different periods of the year on serum LH and FSH concentrations in (a) 6 control intact females, (b) 5 females ovariectomized in September (OVX 1), and (c) 5 females ovariectomized in March (OVX 2). Values are mean ± s.e.m. The arrows indicate the time of ovariectomy.

Fig. 5. Seasonal variations in peak concentrations of LH at (a) 15 min and (b) 2 h after LHRH. Values are mean $\pm$ s.e.m. for 6 control (●—●), 5 OVX 1 (○---○) and 5 OVX 2 (▲---▲) females. The arrows indicate the time of ovariectomy.

Discussion

The radioimmunoassay system developed for sheep and fox FSH can also be used to measure hare FSH, in contrast to a rabbit-antirabbit FSH system previously tested, which produced non-parallel inhibition curves (M. Blanc, M. Meunier & J. C. Poirier, unpublished). As shown in a previous paper, in intact doe hares, there is a clear seasonal variation in LH basal concentrations, which remain undetectable during the anoestrus (September to January) and are maximum from March to July (Caillol *et al.*, 1986). The pituitary LH response to LHRH is also maximum from March to July, and, from January to August, a 2nd peak of LH appears 2 h after injection, in ovulating as well as in non-ovulating females. In contrast, FSH concentrations remain around basal values and the profile of FSH release during the 5 h following LHRH injection is not modified throughout the year; during the breeding season, a 2nd rise in FSH concentrations occurs 24 h after LHRH injection, in relation to the occurrence of ovulation (ovulating *vs* non-ovulating hares: 107 ± 18 ng/ml, $n = 22$, and 32 ± 10 ng/ml, $n = 10$) (Caillol *et al.*, 1986). A similar profile of FSH has been described in the rabbit after mating (Osteen & Mills, 1979; Meunier *et al.*, 1983).

To our knowledge, few data are available concerning seasonal variations of LH and FSH in induced ovulators. In another hare species, *Lepus americanus*, Davis & Meyer (1972) have shown an increase in pituitary content of both gonadotrophins and in serum LH concentrations during the breeding season. In the ferret, Donovan & ter Haar (1977) described higher plasma concentrations of FSH in anoestrous than in oestrous females, while LH values did not differ significantly, and a greater gonadotrophin response to LHRH in anoestrous than in oestrous females. In seasonal breeders with spontaneous ovulation, there is a discrepancy in the results concerning seasonal

variation of FSH. In the mare, there was no seasonal variation in FSH pituitary content or plasma levels (Turner *et al.*, 1979; Hart *et al.*, 1984; Silvia *et al.*, 1986). However, LHRH-induced release of FSH decreased as the breeding season approached (Silvia *et al.*, 1987). In the ewe, the results are conflicting: according to Walton *et al.* (1977) there was no seasonal variation in plasma FSH concentrations, although Findlay & Cumming (1976) and McNatty *et al.* (1984) reported a seasonal variation of plasma FSH which could be related to the seasonal variation in the pituitary content of FSH described by Thimonier & Mauléon (1970).

After ovariectomy in the hare, short-term changes in LH basal concentrations are quite different for the two seasons of ovariectomy: the LH rise, which is immediate when ovariectomy is performed during the breeding season (OVX2), is delayed when ovariectomy is performed during the seasonal anoestrus (OVX1). This suggests a negative feed-back effect of ovarian secretions on LH basal concentrations only during the breeding season. In long-term ovariectomized females, the evolution of LH is similar to that in intact females, with significantly increased values during the breeding season, and low levels during the next anoestrus, suggesting a direct central effect of season, and probably of photoperiod on the regulation of LH secretion, as already described in the snow-shoe hare (Davis & Meyer, 1973), in the mare (Garcia & Ginther, 1976), and in the ewe (Karsch *et al.*, 1984). The increased sensitivity to oestradiol negative feed-back during seasonal anoestrus is well documented in the ewe (Karsch *et al.*, 1984) and in the goat (Mori *et al.*, 1987). In the brown hare, a differential role of oestradiol control upon LH secretion remains to be demonstrated.

In ovariectomized brown hares, the seasonal changes in LH release after LHRH are less important than those of the basal concentrations. In females ovariectomized during the seasonal anoestrus, the rise in the pituitary LH response to LHRH, which begins as early as October, suggests a negative feed-back effect of ovarian secretions during the anoestrus. From April to September, in both groups of ovariectomized females, the lower peak level of LH 15 min after LHRH and the absence of the 2nd LH peak 2 h after LHRH suggest a positive feed-back effect of ovarian secretions during the breeding season, allowing an enhanced duration of LH secretion after LHRH in intact females. A possible role of periovulatory progesterone and/or oestrogens (released after the first LH rise) in the induction of this 2nd LH peak can be suspected (Caillol *et al.*, 1986).

Whatever the season, ovariectomy is followed by an immediate rise in FSH basal concentrations; in the long term, there are no more changes in FSH concentrations. Similar results are obtained concerning the evolution of the pituitary FSH response to LHRH after ovariectomy. This suggests a negative control of the ovary throughout the year. During the seasonal anoestrus, the quiescent ovary could be responsible for the synthesis of oestradiol and inhibin, accounting for the immediate FSH rise after ovariectomy performed in September. The 2nd rise in FSH concentrations observed during the breeding season in intact females 24 h after LHRH never appears in ovariectomized females, and could be related to the completion of ovulation, as already suggested. In the rabbit, the post-ovulatory surge of FSH is not mediated by LHRH, but might be triggered by ovarian factors other than oestradiol (Mills *et al.*, 1983; Mills & Copland, 1983).

Few data are available concerning the relative roles of season and ovarian secretions on FSH regulation. In the snowshoe hare, a rise in pituitary FSH content occurred only when ovariectomy was performed during the breeding season (Davis & Meyer, 1973). The secretion of FSH appeared to be seasonal in the ovariectomized mare (Freedman *et al.*, 1979) which exhibited fewer peaks in FSH concentrations in winter than in summer, even though mean FSH values did not differ between seasons (Thompson *et al.*, 1987). As in the brown hare, short-term changes in FSH concentrations in ewes ovariectomized during the anoestrous or breeding season did not differ significantly; however, in the ewe, but not in the hare, season influenced FSH secretion over longer periods, increasing it at the onset of the breeding season (Montgomery *et al.*, 1987).

For both gonadotrophins, basal levels and pituitary response to LHRH remain higher for females ovariectomized during the seasonal anoestrus (OVX1) than for females ovariectomized during the breeding season (OVX2). This could be due to a long-term modulating effect of

increased ovarian secretions before the ovariectomy performed during the breeding season; this effect would not appear in 6-months-old females ovariectomized during anoestrus.

Our results support the hypothesis that, in the hare, the regulation of LH secretion involves a direct photoperiodic drive responsible for a seasonal rhythm of basal LH release, independent of ovarian influences, and a negative ovarian feed-back which modifies LH concentrations during the breeding season. In intact hares, the enhanced LH response after LHRH during the breeding season is related to an acute positive effect of ovarian secretions. The regulation of FSH is less dependent on seasonal influences and remains under negative ovarian control throughout the year.

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