Plasma melatonin in ewes after ovariectomy

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Summary. Six ewes were ovariectomized in late November and kept in natural light with 5 intact controls. LH levels were monitored approximately weekly. On Days 16 and 58 after ovariectomy plasma melatonin was determined at 3-h intervals for 24 h in all the ewes. Plasma melatonin concentrations were greater in the ovariectomized ewes than in the controls especially at 16 days when total 24 h secretion was almost 3 times that in the control ewes. These data indicate that, in the ewe, the gonads exert an inhibitory influence on melatonin secretion.

Introduction

It is well established that the seasonal pattern of reproductive activity in many species is controlled by the photoperiod (Turek & Campbell, 1979; Lincoln & Short, 1980; Follett & Follett, 1981). Photoperiodicity in the sheep was demonstrated by Yeates in 1949. In sheep pinealectomy abolishes some photoperiod-related hormonal changes (Barrell & Lapwood, 1979; Brown & Forbes, 1980; Karsch, Bittman & Legan, 1981), and denervation of the pineal gland (by removal of the superior cervical ganglia) eliminates the ability to respond to artificial photoperiods (Lincoln, 1979). Evidence of pineal involvement in the ovine reproductive cycle has also been furnished by measurement of peripheral melatonin levels. Circulating melatonin in the ewe shows a diurnal rhythm, values being elevated during darkness and low in continuous light (Rollag & Niswender, 1976; Kennaway, Frith, Phillipou, Matthews & Seamark, 1977). Moreover, the duration of melatonin secretion may reflect the length of darkness (Rollag, O'Callaghan & Niswender, 1978). Arendt, Symons & Laud (1981) have shown that when intact Suffolk-cross ewes are kept in natural light there is often a bimodal pattern of melatonin secretion during oestrus (and short days), but only a single peak during the dark phase in anoestrus (and long days).

Several reports indicate that, in addition to photoperiod, gonadal steroids influence pineal synthesis or secretion of melatonin (Weiss & Crayton, 1970; Cardinali, Nagle & Rosner, 1974; Ozaki, Wurtman, Alonso & Lynch, 1978; Wilkinson & Arendt, 1978; Johnson, Vaughan, Richardson, Petterborg & Reiter, 1982), although the oestrous cycle does not appear to modify plasma melatonin values in the ewe (Rollag *et al.*, 1978; Arendt *et al.*, 1981). In an investigation into factors controlling pineal melatonin secretion in the ewe we have studied the effect of gonadectomy on plasma melatonin levels during 24 h in different (natural) photoperiods, as well as monitoring LH levels in daytime plasma.

Materials and Methods

Animals

Suffolk-cross ewes from the same breeding flock were used. The animals were 8 months of age at the beginning of the experiment. Diet consisted of 2 kg hay and 1 kg ewe and lamb nuts (B.P. Nutrition (U.K.) Ltd, Witham, Essex) per ewe per day. All diets were low in copper content (< 8

p.p.m.). Animals were kept in an outdoor paddock under natural light conditions, and handled frequently in an attempt to avoid experimental stress. Six animals were ovariectomized, and 5 remained intact to serve as a control group.

Sampling

For melatonin determinations blood was sampled from the jugular vein by using vacutainer tubes (lithium-heparin coated, Becton Dickinson Ltd, Cowley, Oxford), at 3-h intervals throughout 24 h, 16 and 58 days after ovariectomy. All dark-phase samples were taken with the help of a dim red torch. All samples were taken according to clock hour, starting and finishing at 09:00 h.

Throughout the study blood was also withdrawn 1 or 2 times a week, normally between 09:00 and 12:00 h, to determine reproductive status by progesterone levels, and also to monitor LH levels. Plasma was either decanted immediately or blood was stored for a maximum of 6 h at 0–4°C before separation, and plasma was then kept frozen at –20°C until assayed.

Ovariectomy

The 6 ewes were fasted for 24 h before induction of general anaesthesia, and under sterile conditions the ovaries were removed through a midline incision into the abdomen. The ovaries were then fixed in 10% buffered formal-saline, embedded in paraffin wax, sectioned and then stained with haematoxylin and eosin. Ovaries from all animals contained corpora lutea. After surgery all ewes received a dose of long-acting antibiotic (Terramycin LA, 200 mg/10 kg body weight (Pfizer, Sandwich, Kent)). All operations were performed between 26 and 28 November 1980.

Assays

Details of the assay procedures used, including specificity, sensitivity and coefficients of variation, are given in the publications cited below for each individual assay.

Progesterone. Progesterone was assayed by radioimmunoassay (Symons, 1973) using antiserum HP/S/53-IIC (Guildhay Antisera, Guildford, U.K.). No significant cross-reactants are found with this antiserum, the major cross-reactants being 11-deoxy corticosterone (0.43%) and corticosterone (0.50%). The sensitivity of the assay, defined as the point distinguished by 2 standard deviations from maximum binding (i.e. zero unlabelled hormone), was 0.2 ng/ml. The mean intra-assay and inter-assay variabilities of 2 plasma samples containing 1.3 and 5.0 ng/ml were 8.4 and 12.3% (CV) respectively. The recovery of tritiated progesterone was 70% (11% CV). The levels were estimated twice weekly (every 3–4 days) to indicate the reproductive status of the intact control ewes. Progesterone levels were also monitored throughout the experiment in ovariectomized ewes to check that no ovarian tissue had been left in the abdomen during surgery.

LH. LH levels were estimated using a slight modification of the method of Martensz, Baird, Scaramuzzi & Van Look (1976). Ovine standards were provided by NIH (NIH-LH-S17) and the antiserum, rabbit anti-ovine LH (ref. R2/1F) was donated by Dr H. M. Fraser, M.R.C. Reproductive Biology Unit, Edinburgh. LH levels were estimated at approximately weekly intervals in all animals. The antiserum was used at a final dilution of 1:60000 and a 24-h preincubation period at 20°C with standards, blanks and samples was employed before the addition of ¹²⁵I-labelled LH (20 000 c.p.m.). A further 24-h incubation period at 20°C was followed by the addition of the second antibody (donkey anti-rabbit; Guildhay Antisera, Guildford) at a dilution of 1:20. After 20 min incubation at 20°C polyethylene glycol was added and incubation was continued for a further 1 h before centrifugation and aspiration of supernatants for gamma counting. The minimum detectable level of LH was 0.5 ng/ml plasma (2 standard deviations from zero standard binding) and cross-reactivity with FSH was less than 0.3%. Interassay coefficients of variation were 20.0% at 1.4 ng/ml plasma and 15.2% at 81.3 ng/ml plasma.

Melatonin. Melatonin was determined by radioimmunoassay as previously described for human plasma (Arendt, Wetterberg, Heyden, Sizonenko & Paunier, 1977). This assay was validated for sheep plasma (Arendt *et al.*, 1981). The major cross-reactant is 6-hydroxymelatonin (0.6%), but the assay is chromatographically specific in sheep plasma (Arendt *et al.*, 1981). The assay sensitivity ranged from 12 to 24 pg/ml. The mean intra-assay and inter-assay variabilities of 3 plasma samples containing 106, 311 and 511 pg/ml were 12.5% and 19.0% (CV) respectively. The assay blank was less than the limit of detection and recovery of tritiated melatonin was 82.6% (1.4% CV). Samples (1 ml) of sheep plasma were extracted and assayed in duplicate using antibody K244. All samples from any one 24-h experiment were measured in the same assay.

Results

Progesterone

Progesterone was undetectable in ovariectomized ewes (assay limit of detection = 0.2 ng/ml), whereas control animals showed the normal 16–18-day oestrous cycles, throughout the experimental period, values ranging from undetectable at the periovulatory period up to 4–5 ng/ml during the 12–14-day luteal phase.

LH

LH levels started to rise within 7 days of ovariectomy and by 14 days values were significantly elevated above control concentrations (unpaired *t* test, $P < 0.001$). The values remained high in the ovariectomized animals throughout the experiment, although a peak was reached around 42 days after operation (Text-fig. 1). Levels then began to fall until at 82 days the LH levels in ovariectomized ewes were significantly lower than those recorded 42 days after operation ($P < 0.01$), but still significantly higher than those observed in the control group of ewes ($P < 0.001$).

Text-fig. 1. Daytime plasma LH levels (mean $\pm$ s.e.m.) in 6 ovariectomized (ovex) and 5 intact (control) Suffolk-cross ewes after ovariectomy on 26–28 November 1980. All values in the ovariectomized ewes (except the first) were significantly higher than the corresponding control level ($P < 0.001$, unpaired Student's *t* test). Analysis of variance of the LH values of ovariectomized ewes with respect to time (excluding the first time point with initial, low, post-ovariectomy values) indicated that significant overall variations were present ($P < 0.01$). LH levels in the ovariectomized ewes at 82, 89 and 93 days after operation (*) were significantly lower than at 42 days ($P < 0.01$, paired Student's *t* test).

Melatonin

In all ewes melatonin secretion was increased in the dark phase of the photoperiod, with low to undetectable levels during the light phase of the cycle (Text-fig. 2). By 16 days after operation melatonin levels during the dark phase were significantly increased at 21:00 and 06:00 h ($P < 0.025$) and 03:00 h ($P < 0.05$) compared with the values in control ewes. Furthermore, the integrated melatonin secretion throughout the 24-h period (estimated from the area under the curve) in ovariectomized animals was significantly ($P < 0.025$) greater (142 ± 28 , mean $\pm$ s.e.m., arbitrary units) than in control ewes (56 ± 18 arbitrary units).

Text-fig. 2. Plasma melatonin levels (mean $\pm$ s.e.m.) in 6 ovariectomized (ovex) and 5 intact (control) Suffolk-cross ewes at 3-h intervals during 24 h when sampled on (a) Day 16 and (b) Day 58 after ovariectomy; * $P < 0.05$; ** $P < 0.025$ compared with control values (unpaired Student's t test). Sunrise and sunset on Day 16 were at 08:02 h and 15:49 h (16:13 h of darkness) and on Day 58 at 7:51 h and 16:34 h (15:17 h of darkness). Two-way analysis of variance (for group effects, time-point effects and interaction) indicated highly significant group effects only on Day 16 (ovex compared with control, $P < 0.01$) and significant group and time-point effects on Day 58 (both: $P < 0.05$).

At 58 days, melatonin secretion was still increased in the ovariectomized animals (195 ± 15 arbitrary units) when compared to control ewes (110 ± 10 arbitrary units) (integrated 24-h secretion, $P < 0.05$). The difference between the ovariectomized and control animals was not as great as that observed at 16 days, due to an increase in secretion and change in the pattern of secretion of melatonin in the control animals (Text-fig. 2).

Discussion

Our ewes were ovariectomized during the last week of November, i.e. well into the breeding season (onset of oestrus normally varies from late September to late October), and all ovariectomized animals showed histological evidence of having ovulated at least once. As expected, all animals showed increased LH levels within 10 days of ovariectomy, reaching a peak about 42 days after operation; levels then fell slightly, but significantly, throughout the rest of the experiment. These observations are in contrast to those of Legan, Karsch & Foster (1977) who noted no change in serum LH in ovariectomized ewes in the course of a year. The observed fall in LH levels could be due to the development of refractoriness of the hypothalamo-hypophysial neurosecretory system, which may become insensitive to the reduced levels of ovarian steroids. However, exposure to the increasing spring daylength itself may induce a decline in gonadotrophin secretion by reducing hypophysial sensitivity to hypothalamic releasing factors or by inhibiting the synthesis and/or release of those releasing factors, possibly via an effect on the pineal gland.

The probable importance of photoperiod in the post-ovariectomy LH rise has been shown in snowshoe hares in which ovariectomy during the non-breeding season, when the gonads were fully regressed, caused no increase in serum LH, although ovariectomy during the breeding season resulted in elevated serum LH levels (Davis & Meyer, 1973). These observations were extended by Turek & Campbell (1979) and Ellis & Turek (1980) who demonstrated the direct effect of photoperiod upon serum LH in castrated golden hamsters. Their results indicated that the effect of castration upon gonadotrophin secretion could be abolished, or overridden, when hamsters were exposed to non-stimulatory photoperiods. Within the time course of our experiment, however, the LH variations within the group of ovariectomized ewes, following the post-gonadectomy rise, are small compared with the difference between ovariectomized and control ewes.

The 24-h experiments performed to determine the effect of gonadectomy on plasma melatonin took place on two occasions when mean circulating daytime LH levels in the ovariectomized and control animals were not significantly different within groups, and are not particularly informative as to the possible interactions of gonadotrophins and the pineal gland. There is, moreover, very little evidence of a direct effect of gonadotrophins on the pineal gland.

The observed increase in total nocturnal melatonin secretion in ovariectomized ewes compared to controls was more marked at 16 days after ovariectomy than that observed at 58 days. The most likely explanation of these observations is that in the intact animals, ovarian steroids and photoperiod are interacting in such a way as to attenuate melatonin secretion: following ovariectomy, an inhibitory influence is removed. At 58 days after ovariectomy total melatonin secretion was still elevated in operated ewes compared to values in control animals, although the melatonin secretion in the control animals was increased. This suggests that, with increasing daylengths, a negative feedback action of ovarian steroids upon melatonin secretion is reduced, and the diurnal patterns of melatonin secretion more directly reflect the entraining effects of the external photoperiod.

It appears that melatonin can advance the seasonal reproductive cycle of the ewe if administered in such a way as to mimic winter or short-day pineal secretion in summer, or long days (Kennaway, Gilmore & Seamark, 1982; Arendt, Symons, Laud & Pryde, 1983). The monitoring of the length of darkness by the duration of night-time melatonin suggests that melatonin could act as an anti- and pro-gonadotrophin. Ovariectomy did not affect the duration of night-time secretion in natural light and it is therefore likely that the absence of gonads does not affect the primary, photoperiodic messenger function of the indoleamine. However, ovarian steroids certainly seem to be involved in the fine control of quantitative aspects of pineal secretion. These results suggest that a more thorough investigation of pineal function during the oestrous cycle of the ewe would be worthwhile.

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