Diurnal Changes in Plasma Melatonin and the Timing of Reproductive Onset in Anestrous Sheep Fed Melatonin

Naohito KUSAKARI, Yuko TAJIMA¹⁾, Shunsuke ITOH, Kazuhiro SEN-NA, Shin SERIKAWA, Tadao HATTA, Mutsuo OHARA, and Yuji MORI¹⁾

Takikawa Animal Husbandry Experiment Station of Hokkaido, Takikawa, Hokkaido 073 and ¹⁾Laboratory of Veterinary Reproduction, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan

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ABSTRACT. Seasonally anestrous Suffolk ewes (n=28) were randomly divided into 5 groups and treated with varying doses of melatonin as follows; Groups C (n=4), M1 (n=6), M2 (n=6), M3 (n=6), M4 (n=6) of ewes were fed pellets containing 0, 1, 2, 3 and 4 mg melatonin, respectively, daily for 60 days from May 17 (Day 0). Following feeding of the pellets at 13.00 hr plasma levels of melatonin rapidly increased reaching the peak values within 30 min, which ranged from 92.0 to 292.7 pg/ml and were highly correlated with the dose of melatonin administered ($r=0.986$, $P<0.01$). Maximum dose of melatonin (4 mg) produced an increase of plasma melatonin similar in magnitude to nocturnal peaks of endogenous secretion. The onset of ovulatory cyclicity, assessed from plasma progesterone profiles, was advanced by melatonin administration. The mean $\pm$ SEM intervals from the commencement of melatonin treatment until the onset of ovulatory cyclicity were 53.0 ± 5.8 , 53.6 ± 2.5 , 42.0 ± 5.6 and 44.3 ± 4.3 days for the Groups M1, M2, M3 and M4, respectively, which were shorter (M1, $P<0.05$; M2, M3 & M4, $P<0.01$) than that for the Group C (72.5 ± 1.4 days). The melatonin treatment also suppressed, in a dose related manner, the rise in plasma prolactin under the lengthening photoperiod. We conclude that the dose-related efficacy of melatonin could be ascribed to the difference in the diurnal profiles of circulating melatonin.—**KEY WORDS:** diurnal change, melatonin, prolactin, seasonal reproduction, sheep.

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The breeding activity of the ewe is known to be under the photoperiodic control [23], and recent studies have revealed that the information on changing daylength is mediated by melatonin secreted from the pineal gland during the dark period [2]. Simulation of short-day patterns of melatonin secretion, by means of timed oral administration of exogenous melatonin, has been demonstrated to induce cyclic ovarian activity and to suppress photoperiodically induced hyperprolactinemia in seasonally anestrous ewes [1, 5, 18]. The daily dose of melatonin used successfully to date varied from 2 to 10 mg per animal [4, 17]. In these previous studies, however, post-feeding rises of plasma melatonin were not carefully examined in comparison with endogenous nocturnal elevation. Consequently the critical melatonin rises over daytime basal levels for induction of short-day responses have yet to be clarified, and it is also unclear as to whether there is any relationship between dose of melatonin and advancement of the onset of cyclic activity. This kind of information would be of practical importance in determining the most cost-effective means of advancing breeding season onset using orally active melatonin.

The present study was therefore conducted to

examine in seasonally anestrous ewes fed different amount of melatonin 1) the diurnal patterns of plasma melatonin, 2) timing of reproductive recrudescence, and 3) long-term changes in plasma prolactin.

MATERIALS AND METHODS

Experimental design: Twenty eight suffolk ewes, ranging from 3–7 years in age and 53–81 kg in body weight, were maintained in outdoor corrals under natural conditions of photoperiod and temperature at the Takikawa Animal Husbandry Experiment Station (43° 35' north latitude). The animals were randomly divided into 5 groups on May 17, 1989 (Day 0): the ewes in the Group C (n=4), M1 (n=6), M2 (n=6), M3 (n=6) and M4 (n=6) were daily given 100 g feed pellets containing 0, 1, 2, 3 and 4 mg melatonin, respectively, at 13.00 hr for 60 days.

Melatonin pellets were prepared as follows. Melatonin solution in ethanol (1, 2, 3 or 4 mg in 2 ml ethanol) was adsorbed onto 100 g lots of pelleted foodstuff (lucerne chaff), and the solvent was allowed to evaporate before feeding. The Group C ewes received the same amount of untreated pellets.

Blood samples were taken by jugular venepunc-

ture using heparine coated vacutainers at 10.00 hr once weekly from Day 0 and twice weekly from Day 28 to Day 75 to trace long-term changes in plasma prolactin and progesterone. Diurnal profiles of plasma melatonin were examined in serial blood samples collected from 20 ewes (4 ewes were randomly chosen from each group) at 15 min to 2 hr intervals for 25 hr starting 10.00 hr on Day 13. Blood sampling during the dark period was carried out under dim red lights (less than 0.3 lux at the height of ewes' head). The plasma samples were obtained by immediate centrifugation and stored at -20°C until assayed for hormone concentrations.

Hormone assays: Progesterone concentrations were analyzed by the double-antibody radioimmunoassay described by Mori *et al.* [8]. Plasma samples (200 μl) were assayed in duplicate following extraction with diethylether. The limit of detection (95% confidence limit of buffer control) was 0.094 ng/ml. Intra- and inter-assay coefficients of variation were 8.4% and 13.7%, respectively.

Melatonin concentrations were measured by the radioimmunoassay described by Mori and Okamura [9] and Mori *et al.* [11]. Plasma (200 μl) was directly assayed, and the limit of detection was 10.3 pg/ml. Intra- and inter-assay coefficients of variation were 7.0% and 16.2%, respectively.

Prolactin concentrations were assayed by the radioimmunoassay described by Mori *et al.* [10]. Plasma (10 μl) was assayed in duplicate, and the limit of detection was 98.1 ng/ml. All the samples were measured in a single assay, and the intra-assay coefficient of variation was 7.0%.

Statistical analysis: Results were expressed as means $\pm$ SEM and Student's *t* test, Cochran-Cox test or Duncan's new multiple range test [16] were used to test for statistical differences among group-means.

RESULTS

Diurnal patterns of plasma melatonin in ewes receiving different doses of melatonin (0–4 mg) at 13.00 hr under natural long days are shown in Fig. 1. Control ewes given vehicle pellets showed a distinct diurnal rhythm of plasma melatonin with the secretory phase being restricted to the night. This nocturnal elevation was also apparent in all the melatonin treated animals, and both the nocturnal peaks and the pre-feeding daytime levels of plasma melatonin were indistinguishable among the 5

Fig. 1. Diurnal changes in plasma melatonin concentrations in ewes fed varying doses of melatonin in the early afternoon (arrow) under the natural long-day photoperiod (May 30). Each point represents the mean $\pm$ SEM of four ewes from the Groups M1 (1 mg melatonin daily, Δ), M2 (2 mg, $\square$), M3 (3 mg, $\bullet$), M4 (4 mg, $\blacktriangle$) and the Group C (0 mg as the control, $\circ$). The shaded area indicates the period of darkness.

groups. Nocturnal melatonin levels averaged 295.4–310.3 pg/ml with no statistical difference due to the treatment.

Following feeding of the melatonin pellets there was a rapid elevation in plasma melatonin, which reached the peak levels within 30 min. Plasma melatonin then gradually declined to less than half of the initial peak value by 5 hr post-feeding and finally merged into the nocturnal elevation of endogenous melatonin secretion. The peak values attained within 30 min after feeding of the melatonin pellets were dose dependent, and averaged 92.0, 139.4, 184.6 and 292.7 pg/ml for the Groups M1, M2, M3 and M4, respectively. The magnitude of increment was almost equivalent to the nocturnal elevation in the Group M4 (4 mg melatonin), while it was about one third in the Group M1 (1 mg). Figure 2 shows the correlation ($r=0.986$, $n=20$, $P<0.01$) between the dose and the post-feeding peak value of plasma melatonin.

Daily administration of melatonin advanced the onset of reproductive activity in seasonally anestrus ewes in all treatments. Occurrence of cyclic ovarian activity in individual ewes, which was assessed by plasma progesterone profiles, is schematically illustrated in Fig. 3. The onset of ovulatory cyclicity was designated to be 3 days prior to the first

Fig. 2. Dose dependent increase of plasma melatonin concentrations 30 min after feeding of 0-4 mg melatonin in seasonally anestrus ewes. Each point represents the mean $\pm$ SEM of four ewes. For further explanation see Fig. 1.

Fig. 3. Occurrence of cyclic ovarian activity (solid bars) in individual ewes fed melatonin daily. Reproductive status was assessed by plasma progesterone profiles: values of greater than 1 ng/ml for two consecutive samples were taken to indicate the onset of ovarian activity.

Fig. 4. Long-term changes in plasma prolactin in ewes given 0 mg (C), 1 mg (M1), 2 mg (M2), 3 mg (M3) and 4 mg (M4) melatonin, respectively for 60 days. Period of melatonin feeding is the horizontal bars. Each point depicts the mean $\pm$ SEM ($n=4-6$).

rise (exceeding 1 ng/ml) of plasma progesterone which was followed by regular changes in progesterone levels during ovulatory cycles. In the Group C two ewes showed an increase of plasma progesterone on Day 72, but the remaining two did not by the end of experiment (Day 75). On the other hand, all the melatonin treated ewes ovulated during the experiment, some as early as Day 25. The average intervals from the start of melatonin treatment to the onset of ovulatory cyclicity were 53.0 ± 5.8 , 53.6 ± 2.5 , 42.0 ± 5.6 and 44.3 ± 4.3 days for the Groups M1, M2, M3 and M4, respectively, which were significantly shorter (M1, $P < 0.05$; M2, M3, M4, $P < 0.01$) than that for the Group C (72.5 ± 1.4 days, calculated by assigning a value of 75 days to the two anovulatory ewes). The higher doses of melatonin tended to induce the earlier resumption of ovulatory cyclicity in the more consistent manner, although the intervals did not differ statistically among the melatonin treated groups. There was an inverse relationship ($r = -0.609$, $n = 28$; $P < 0.001$) between the dose of melatonin (x mg) and the interval (y days) to the onset of ovulatory cyclicity (slope, -6.23 ; Y intercept, 65.05).

Long-term changes in plasma prolactin are shown in Fig. 4. Prolactin levels in the Group C ewes showed a gradual rise ($P < 0.01$) from 107.8 ± 3.8 ng/ml (Day 0) to 386.9 ± 89.7 ng/ml (Day 65) along with increasing daylength. In contrast, plasma prolactin in the Groups M2, M3 and M4, but not in the Group M1 which showed similar rise to the Group C, remained low within a range similar to those

found on Day 0 throughout the period of melatonin treatment (Day 0–59). Overall mean values of plasma prolactin during the melatonin treatment for the Groups M2 (132.3 ± 7.7 ng/ml, $n=48$), M3 (118.0 ± 7.4 ng/ml, $n=48$) and M4 (116.5 ± 4.8 ng/ml, $n=48$) were lower (M2, $P<0.05$; M3 and M4, $P<0.01$) than those for the Groups M1 (180.2 ± 18.8 ng/ml, $n=48$) and C (174.1 ± 16.8 ng/ml, $n=32$).

DISCUSSION

The present study has demonstrated that increasing doses of melatonin caused parallel increases in the plasma melatonin levels in the period after administration in ewes. The melatonin administration of any dose did not influence the endogenous melatonin secretion, and the nocturnal melatonin peaks were virtually identical among the groups. The results support the view that regulatory system for melatonin secretion lacks a negative feedback loop [4, 6, 11].

The post-feeding increment of plasma melatonin was similar in magnitude to that found by Kennaway and Seamark [7] in which 2 mg melatonin induced an increase of about 100 pg/ml over daytime basal melatonin levels, but was considerably lower than those reported by others [1, 4, 5], e.g., 500–800 pg/ml increases following 2–3 mg administration. The reason for this inconsistency is unclear, but it seems unlikely that this is due to the breed difference since different results were obtained even in the same Merino crossbred ewes, i.e., Kennaway and Seamark [7] vs Kennaway *et al.* [5]. Amounts of pelleted foodstuff on which melatonin was adsorbed might have influenced the rate of melatonin absorption from the rumen; Kennaway and Seamark [7] and we used 100 g per animal whereas Kennaway *et al.* [5] 40 g. Likely variation in rumen fauna as well as flora should also be considered.

Daily melatonin treatment was effective in advancing the onset of ovarian activity in seasonally anestrus ewes. With increasing the amount of melatonin ewes tended to show earlier commencement of ovulatory cyclicity, although there was no statistical differences in the time of onset between the different treatment groups. With daily dose of 1 mg ovarian responses of individual animals were quite variable, while more consistent results were obtained with higher doses of melatonin. Similar results were reported in the previous studies in which early onset of breeding activity was success-

fully induced with daily doses of 2–3 mg melatonin [1, 4, 5].

The melatonin treatment suppressed plasma prolactin levels in a dose dependent manner. Prolactin secretion by the pituitary gland shows a distinct seasonal fluctuation in domestic ruminants [3, 13, 20, 22]. Since photoperiod is one of the main environmental factors responsible for this change [10], prolactin is the hormone providing a convenient physiological index how the animal is responding to the prevailing photoperiod [14]. Changes in daylength is mediated by the pineal gland through its secretory pattern of melatonin, and thus prolactin secretion could be controlled by manipulating the peripheral melatonin levels [9]. In fact, various melatonin treatments resulted in a suppression of prolactin secretion in ewes kept under long days [5, 6, 12, 14, 15, 18].

It was shown in pinealectomized hamsters that isolated melatonin administrations are capable of conveying photoperiodic information with one of them acting as the signal of dusk [19, 21]. The present results imply that the afternoon rises of plasma melatonin with sufficient magnitude just after the feeding of melatonin pellets are perceived by the ewe as the onset of extended dark phase, whereas smaller increases as compared with endogenous secretion are insufficient to induce short-day responses consistently. Therefore, the dose-dependency of melatonin effects on ovarian activity and prolactin secretion should be ascribed to differences in the diurnal patterns of circulating melatonin.

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