Effect of melatonin implants on reproduction in the male silver fox (*Vulpes vulpes*)

M. Forsberg, J. A. Fougner*, P. O. Hofmo† and E. J. Einarsson*

Departments of Clinical Chemistry and Obstetrics & Gynaecology, College of Veterinary Medicine, Swedish University of Agricultural Sciences, P.O. Box 7038, S-750 07 Uppsala, Sweden;

*Norwegian Fur Breeders Association, P.O. Box 145, 0589 Oslo 5, Norway; and †Department of Reproductive Physiology & Pathology, Norwegian College of Veterinary Medicine, P.O. Box 8146, Dep., 0033 Oslo 1, Norway

Summary. In June 1987, when the testes were fully regressed, 5 males were given s.c. implants of 40 mg melatonin. The same treatment was repeated in August and October 1987. Five males served as controls. Plasma concentrations of melatonin increased significantly in treated males and were still elevated at the end of the study in April 1988. The changes in testicular volume and blood plasma concentrations of testosterone in response to GnRH indicated that melatonin administration promoted testicular development. However, testicular regression was observed earlier in treated than control animals, perhaps because of refractoriness to melatonin or to a down-regulation of melatonin receptors. Semen was collected and frozen in November 1987, 2 months ahead of the natural breeding season, from the melatonin-treated males, and 10 blue fox vixens were inseminated the following breeding season: 9 vixens conceived, and the average litter size was 7.6 ± 0.5 .

The results demonstrate that melatonin treatment initiated during exposure to naturally long days (a) promotes testicular development in a way similar to an artificial short photoperiod and (b) may induce a refractory condition after an extended period of treatment.

Keywords: light; melatonin; reproduction; silver fox; male

Introduction

The silver fox (*Vulpes vulpes*, a colour mutant of the red fox) is classified as a seasonal short-day breeder. Sexual development occurs during the autumn, with a breeding season during winter/early spring (January to late March) depending on latitude (Lloyd & Englund, 1973; Maurel & Boissin, 1981). Male silver foxes are sexually active for a period of approximately 6 weeks each year. The testes undergo photo-mediated changes in activity (Lloyd & Englund, 1973; Forsberg *et al.*, 1989). The pineal gland is an important mediator of the photoperiodic time cues through which seasonal breeders respond to the annual cycle in daylength (for review see Arendt, 1986). The pineal secretes melatonin, the blood concentrations of which increase during the hours of darkness. By giving animals exogenous melatonin it is possible to interfere with the time-keeping mechanism (blue fox: Smith *et al.*, 1987; silver fox: Connor, 1988). The process by which exogenous melatonin regulates hypothalamic–pituitary gonadal function can be interpreted in different ways (Hastings *et al.*, 1985; Arendt, 1986; Reiter, 1987; Arendt *et al.*, 1988). It is the duration of high levels of melatonin throughout the 24 hours that is critical. Alternatively, high levels of melatonin coincide with a circadian period of sensitivity to the compound and produce the gonadal response.

In the present study silver fox males were provided with melatonin implants, starting during the summer when the days are long, to determine whether the breeding cycle could be advanced and

reproductive activity maintained into the normal breeding season. In addition, we wanted to find out whether fertile semen could be collected and frozen for use in a subsequent breeding cycle.

Materials and Methods

Animals and experimental design. Ten silver fox males, aged 1–3 years, were housed individually under natural day-light conditions at the Swedish University of Agricultural Sciences, Uppsala (latitude: 59°50'N; longitude: 17°50'E) from May 1987 until the end of April 1988. The animals were given water *ad libitum* and fed a standard ration wet feed. Melatonin implants (Wildlife Laboratories Inc., Fort Collins, CO, USA) (10 mg; length 9 mm, width 2 mm, surface area 65 mm²) were embedded beneath the skin over the left chest wall while the animals were anaesthetized with ketamine (10 mg/kg) (Ketalar, 50 mg/ml: Parke-Davis, Raritan, NJ, USA). Half of the (5) males were implanted with 40 mg melatonin on 1 June 1987 (when the testes were fully regressed) and again on 4 August and 5 October 1987. All implants remained in place until the end of the experiment. The other 5 males served as controls.

Measurement of testicular volume. The volume of the testes was estimated at approximately monthly intervals. Both testes were stabilized in the scrotum by applying a firm pressure on top of the tissue and then placed in a beaker filled with water. The volume of water displaced was measured and taken as the testicular volume. Repeated measurements gave a coefficient of variation of 5% (larger volumes in the breeding season) to 10% (smaller volumes in the regressed state).

Semen collection and treatment. During the natural breeding season in February and March 1987, before the experiment started, semen was collected from all 10 males to verify that their testicular activity was normal. In November 1987, semen was collected for freezing and subsequent artificial insemination (AI). Techniques for semen collection, evaluation and freezing have previously been reported (Aamdal, 1972; Hofmo, 1988; Forsberg *et al.*, 1989).

Detection of oestrus and insemination. Time of insemination was determined by the farmer based on vulval swelling and electrical resistance of the vagina (Möller, 1980). All vixens were inseminated twice at 24-h intervals with semen from the same male as described by Fougner *et al.* (1973), with approximately 100 × 10⁶ progressively motile spermatozoa.

Ten blue fox vixens, aged 1–4 years, were inseminated from 25 March to 29 March 1988 to test the fertilizing ability of the frozen semen.

Blood sampling. Once each month, between 11:00 and 15:00 h, animals were sedated (2 mg acepromazine/kg (Plegicil, 10 mg/ml: Agrivet AB, Uppsala, Sweden) and 4 cephalic vein blood samples were taken at 20–30-min intervals. Then 20 µg GnRH (Nialutin: Novo Industry A/S, Bagsvaerd, Denmark, 20 µg/ml) were administered i.m. immediately after taking the second blood sample to monitor testicular testosterone response to LH release (Hopkinson *et al.*, 1974; Forsberg *et al.*, 1989). Plasma was stored at –20°C. Melatonin was assayed from blood samples taken before the GnRH injection.

Hormone assays. Plasma concentrations of testosterone were determined by a radioimmunoassay previously validated for use in the silver fox (Forsberg *et al.*, 1989). The intra-assay coefficients of variation, calculated from the precision profiles of 5 different assays, were below 13% in the range of 3.5 to 34.7 nmol/l. The inter-assay coefficients of variation (%) for quality control samples were 8.6 (mean = 9.2 nmol/l, *n* = 13), 8.0 (mean = 17.4 nmol/l, *n* = 13) and 8.6 (mean = 25.5 nmol/l, *n* = 11).

Testosterone released during the first 60 min after GnRH injection was assessed from the areas under the release curves according to the formula:

$$\text{Hormone (H) response} = \Sigma((H_i + H_{i+30})/2) \times 30 \text{ min}$$

where *i* = 0 or 30.

The assay of melatonin used an antiserum raised against N-succinyl-5-methoxy tryptamine (Rollag & Niswender, 1976) with a [³H]melatonin tracer as previously described and validated for use in the blue fox (Forsberg & Madej, 1987). Serial dilutions of silver fox plasma containing a high melatonin concentration produced a dose–response curve parallel to the standard curve. The intra-assay coefficient of variation was 20% at 84 pmol/l and remained below 15% for concentrations exceeding 168 pmol melatonin/l. The coefficients of variation (%) for two pooled plasma samples of silver fox origin, estimated in 5 different assays, were 15.5 (mean = 346 pmol/l) and 11.9 (mean = 649 pmol/l).

Hormone concentrations of testosterone and melatonin represent the means of duplicate and triplicate determinations.

Statistical methods. Statistical analysis of the data was carried out with the Statgraphics (STSC, Inc., Rockville, MD, USA) statistical package using analysis of variance. Scheffé's method for multiple comparisons was used to test significances for between-treatment groups. Within-treatment group comparisons were made by the LSD test. All statistical tests having a *P* value of <0.05 were considered significant. Means are expressed as ± s.e.m.

Results

The overall mean plasma concentration of melatonin was significantly increased in samples collected at monthly intervals from June 1987 to April 1988 from the melatonin-implanted males (experimental group, 917 ± 56 pmol/l; control group, 120 ± 15 pmol/l).

The size of the testes and the release of testosterone in response to GnRH in the melatonin-implanted and control animals changed significantly with time (Figs 1a, b).

Fig. 1. Seasonal changes in (a) testicular volume and (b) testosterone release after GnRH challenge of control (■) and melatonin-treated (□) animals (N = 5). The arrows depict the dates of melatonin administration and the horizontal bar the natural breeding season. Values are mean $\pm$ s.e.m.

The testes of the melatonin-treated animals increased significantly in volume within 30 days of the first implantation in June, reached maximal values in January, and thereafter declined significantly until the end of the study in April. The testes of the control animals remained regressed until

September, increased significantly and reached maximal values in February, 1 month after the melatonin-treated males, and then declined significantly until April.

In melatonin-treated animals testosterone release reached significant peak values in November, December and January. In control animals values peaked significantly in February and March.

There was a significant time $\times$ group effect, indicating that the long-term changes in testicular volume and testosterone secretion differed between melatonin-treated and control animals. Differences between groups in mean testicular volume and testosterone release became evident within 3 and 5 months of the first implantation.

During the 2nd week of November 1987 it was possible to collect normal semen from all melatonin-treated animals but not from the control animals. Semen was collected and frozen on 4 consecutive days during the 4th week of November 1987. The volume and pre-freezing progressive motility of the semen from 13 ejaculates averaged 0.43 ± 0.16 ml and $88 \pm 2\%$, respectively. Sperm abnormalities were less than 10%.

Of the 10 blue fox vixens inseminated the following breeding season, 9 conceived, and the average litter size from 7 of the females was 7.6 ± 0.5 (range 5–10; 2 vixens killed their cubs at parturition).

Discussion

The results from the present investigation illustrate that sexually inactive silver fox males exposed to high concentrations of melatonin during a period of naturally long days beginning in June prematurely show the reproductive changes associated with testicular redevelopment. Similarly, a premature onset of reproductive activity follows exposure to artificial short days when treatment is begun in June (Forsberg *et al.*, 1989). This is consistent with earlier reports for sheep in which melatonin implants induced a short-day response provided that the animals had been exposed to a period of long days when artificial light was used, or that the treatment was begun around or after the summer solstice if natural light was used (Nowak & Rodway, 1985; Lincoln & Ebling, 1985; English *et al.*, 1986). In a similar experiment with melatonin implants in blue fox males (*Alopex lagopus*) in August no clear effects on testicular redevelopment were observed (Smith *et al.*, 1987). Although melatonin concentrations were significantly elevated for 6 months in that study, concentrations declined rapidly after implantation, and so may not have been high enough to induce testicular redevelopment.

The fact that the males implanted with melatonin in the present study did not have a prolonged period of testicular activity in response to the continued exposure to high melatonin concentrations during the spring months was unexpected. Advanced testicular development and premature decline in testicular function in silver fox males after melatonin implantation has also been reported by Connor (1988). Animals implanted once with 8 or 12 mg melatonin in July showed testicular regression in February, 1–2 months before control animals (Connor, 1988). This may indicate the presence of an endogenous rhythm of breeding activity (Pengelley, 1974; Gwinner, 1981), which would allow the breeding season to be shifted in time but not lengthened. This contradicts the results obtained when sexually active males were exposed to high concentrations of melatonin (unpublished results) or subjected to short days (Forsberg *et al.*, 1989) from the beginning of the breeding season in February until June. These animals showed testicular activity in June virtually identical to that seen at the height of the breeding season 3 months previously by means of histology, testosterone secretion and semen production.

An alternative interpretation is that males became insensitive or refractory to the formerly inductive melatonin signal after an extended period of exposure to high melatonin concentrations. Loss of response to a formerly stimulatory melatonin signal has been reported by Lincoln & Ebling (1985) who observed that Soay rams became refractory to long-term treatment with constant release implants of melatonin. Nett & Niswender (1982) observed that daily injections of melatonin

into ewes late in the breeding season were only temporarily effective in preventing the onset of anoestrus, and Karsch *et al.* (1986) and Poulton *et al.* (1987) reported that ewes treated with melatonin eventually became unresponsive to a formerly inductive melatonin signal. The cause of the reproductive suppression needs further investigation. However, desensitization of the target tissues of the pineal to melatonin or down-regulation of melatonin receptors should not be excluded as an explanation of the reproductive suppression (Bittman, 1978; Reiter, 1980; Arendt, 1986).

In the present study we demonstrated that semen collected and frozen during an artificially induced period of testicular activity possessed fertilizing ability. Although the results must be viewed with some caution because of the small number of inseminations, the group of vixens inseminated with the semen collected and frozen before the natural breeding season appeared to have a normal conception rate and normal litter size. This conclusion is supported by observations made by Hofmo (1988) who reported a conception rate of 87% and an average litter size of 7.7 when 207 blue fox vixens were inseminated with silver fox semen collected and frozen during the natural breeding season.

Advancing the period of testicular activity should facilitate artificial insemination in foxes. Implantation of melatonin is a practical method for manipulating the breeding cycle under field conditions (although the method may be unacceptable in some countries because of animal protection legislation). Freezing semen before the natural breeding season will allow progeny testing of treated males and advance the integration interval of breeding schemes by 1 year. However, although the constant stimulation of melatonin implants can advance sexual activity, it cannot maintain it and so the overall reproductive capacity of the individual male will not increase. If the decline in testicular function is a consequence of the long-term treatment with melatonin this problem may be circumvented by alternating monthly periods of melatonin treatment with periods of no treatment (Chemineau *et al.*, 1988).

We thank Dr Gordon Niswender for the melatonin antibody; and the Norwegian and Swedish Fur Breeders Associations for financial support.

References

- Aamdal, J. (1972) Investigation in the reproduction in the blue fox. In *Riproduzione Animale e Fecondazione Artificiale*, pp. 1–6. Edizione Agricole, Bologna.
- Arendt, J. (1986) Role of the pineal gland and melatonin in seasonal reproductive function in mammals. *Oxford Rev. Reprod. Biol.* **8**, 266–320.
- Arendt, J., Symons, A.M., English, J., Poulton, A.L. & Tobler, I. (1988) How does melatonin control seasonal reproductive cycles? *Reprod. Nutr. Develop.* **28** (2B), 387–397.
- Bittman, E.L. (1978) Hamster refractoriness: the role of "insensitivity of pineal target tissue". *Science, NY* **202**, 648–650.
- Chemineau, P., Pelletier, J., Guerin, Y., Colas, G., Ravault, J.P., Toure, G., Almeida, G., Thimonier, J. & Ortavant, R. (1988) Photoperiodic and melatonin treatments for the control of seasonal reproduction in sheep and goats. *Reprod. Nutr. Develop.* **28** (2B), 409–422.
- Connor, M.L. (1988) Melatonin as a furring enhancer: Effects on growth rate, feed consumption and reproduction in the silver fox. In *Biology, Pathology and Genetics of Fur Bearing Animals*, pp. 437–443. Eds B. D. Murphy & D. B. Hunter. Canada Mink Breeders Association, Rexdale, Ontario, Canada.
- English, J.E., Poulton, A.L., Arendt, J. & Symons, A.M. (1986) A comparison of the efficiency of melatonin treatments in advancing oestrus in ewes. *J. Reprod. Fert.* **77**, 321–327.
- Forsberg, M. & Madej, A. (1987) Validation of a direct radioimmunoassay of melatonin in the blue fox. *Acta vet. scand.* **28**, 73–79.
- Forsberg, M., Fougner, J.A., Hofmo, P.O., Madej, M. & Einarsson, E.J. (1989) Photoperiodic regulation of reproduction in the male silver fox (*Vulpes vulpes*). *J. Reprod. Fert.* **87**, 115–123.
- Fougner, J.A., Aamdal, J. & Andersen, K. (1973) Intra-uterine insemination with frozen semen in the blue fox. *Nord. VetMed.* **25**, 144–149.
- Gwinner, E. (1981) Circannual clocks: their dependence on the circadian system. In *Biological Clocks in Seasonal Reproductive Cycles*, pp. 153–169. Eds B. K. Follett & D. E. Follett. Wright, Bristol.
- Hastings, M.H., Herbert, J., Martensz, N.D. & Roberts, A.C. (1985) Annual reproductive rhythms in mammals: mechanisms of light synchronization. *Ann. N.Y. Acad. Sci.* **453**, 182–204.
- Hofmo, P.O. (1988) *Studies on cryopreservation of fox spermatozoa and evaluation of the fertilizing capacity of frozen/thawed silver fox spermatozoa*. Dr. Scient. Thesis, Norwegian College of Veterinary Medicine, Oslo.
- Hopkinson, C.R.N., Pant, H.C. & Fitzpatrick, R.J. (1974) Release of LH and FSH in the normal intact

- ram by synthetic LH-RF and the effect of pretreatment with testosterone propionate. *J. Reprod. Fert.* **39**, 135–139.
- Karsch, F.J., Bittman, E.L., Robinson, J.E., Yellon, S.M., Wayne, N.L., Olster, D.H. & Kaynard, A.H. (1986) Melatonin and photorefractoriness: loss of response to the melatonin signal leads to seasonal reproductive transition in the ewe. *Biol. Reprod.* **34**, 265–274.
- Lincoln, G.A. & Ebling, F.J.P. (1985) Effect of constant release implants of melatonin on seasonal cycles in reproduction, prolactin secretion and moulting in rams. *J. Reprod. Fert.* **73**, 241–253.
- Lloyd, H.G. & Englund, J. (1973) The reproductive cycle of the red fox in Europe. *J. Reprod. Fert., Suppl.* **19**, 119–130.
- Maurel, D. & Boissin, J. (1981) Plasma thyroxine and testosterone levels in the red fox (*Vulpes vulpes* L.) during the annual cycle. *Gen. comp. Endocrinol.* **43**, 402–404.
- Möller, O. (1980) Elektrisk brunstdiagnostikk hos blårev, sølvrev og mårhund—et nytt hjelpemiddel i paringssesongen. *Norsk Pelsdyrblad* **54**, 591–595.
- Nett, T.M. & Niswender, G.D. (1982) Influence of exogenous melatonin on seasonality of reproduction in sheep. *Theriogenology* **17**, 645–652.
- Nowak, R. & Rodway, R.G. (1985) Effect of intravaginal implants of melatonin on the onset of ovarian activity in adult and prepubertal ewes. *J. Reprod. Fert.* **74**, 287–293.
- Pengelley, E.T. (1974) *Circannual Clocks*. Academic Press, New York.
- Poulton, A.L., English, J., Symons, A.M. & Arendt, J. (1987) Changes in plasma concentration of LH, FSH and prolactin in ewes receiving melatonin and short-photoperiod treatments to induce early onset of breeding activity. *J. Endocr.* **112**, 103–111.
- Reiter, R.J. (1980) The pineal and its control of reproduction in mammals. *Endocrine Rev.* **1**, 109–131.
- Reiter, R.J. (1987) Mechanisms of control of reproductive physiology by the pineal gland and its hormones. In *Advances in Pineal Research*, pp. 109–125. Eds R. J. Reiter & F. Fraschini. John Libbey & Company Ltd, London.
- Rollag, M.D. & Niswender, G.D. (1976) Radioimmunoassay of serum concentrations of melatonin in sheep exposed to different lighting regimens. *Endocrinology* **98**, 482–498.
- Smith, A.J., Mondain-Monval, M., Andersen Berg, K., Simon, P., Forsberg M., Clausen, O.P.F., Hansen, T., Möller, O.M. & Scholler, R. (1987) Effects of melatonin implantation on spermatogenesis, the moulting cycle and plasma concentrations of melatonin, LH, prolactin and testosterone in the male blue fox (*Alopex lagopus*). *J. Reprod. Fert.* **79**, 379–390.

Received 19 June 1989