Melatonin administration enhances the reproductive capacity of young rams under a southern Mediterranean environment

Mourad REKIK,1 Rahma TABOUBI,2 Imene BEN SALEM,2 Younes FEHRI,3 Cyrine SAKLY,4 Narjess LASSOUED5 and Muhi Eddine HILALI1

1International Centre for Agricultural Research in the Dry Areas (ICARDA), Amman, Jordan, 2Ecole Nationale de Médecine Vétérinaire, Sidi Thabet, 3CEVA Santé Animale, Tunis, 4Institut Supérieur Agronomique de Chott Meriem, Sousse and 5Laboratoire de Recherche Productions Animales et Fourragères, Institut National de la Recherche Agronomique de Tunisie (INRAT), Ariana, Tunisia

ABSTRACT

This study tested the effect of melatonin treatment, initiated in late February on reproductive traits of young rams. A total of 14 young Barbarine rams were used. Seven animals were treated with three melatonin subcutaneous implants (Melatonin) on 28 February while the remaining rams remained untreated (Control). After 60 days of melatonin administration, scrotal circumference reached average values of 32.1 ± 1.54 and 29.5 ± 1.0 cm for Melatonin and Control animals, respectively (P < 0.05). Semen characteristics did not differ between groups; melatonin treatment tended (P = 0.091) to increase sperm concentration 60 days after implantation when means reached 5.87 ± 0.703 and 4.61 ± 0.654 × 10^9 spermatozoa/mL for Melatonin and Control rams, respectively. Melatonin treatment significantly affected total activity time, number of lateral approaches and mount attempts in comparison to controls. During a 6-h sampling period, mean plasma testosterone concentrations increased as a result of melatonin treatment (P < 0.001) and testosterone pulse frequency averaged 3.45 ± 2.24 and 1.25 ± 1.0 (P = 0.086) for Melatonin and Control rams. Data clearly suggest that abrupt treatment of young rams with melatonin implants in winter is sufficient to improve reproductive traits.

Key words: libido, melatonin, semen, testosterone, young rams.

INTRODUCTION

Mediterranean sheep breeds show an earlier onset of the breeding season, compared to other breeds located at higher latitudes, even when both are subjected to the same photoperiodic treatment (Martin et al. 1999). The Mediterranean breeds have short seasonal anoestrus and management factors, such as proper use of the ‘ram effect’ or strategic use of feed supplements may prove effective in limiting periods of reproductive inactivity (Lindsay 1996). However, age of the animals increases sensitivity to anoestrus in both sexes. Under a southern Mediterranean environment, maiden ewes were reported to be strict seasonal animals unlike their adult counterparts (Khaldi 1984). Furthermore and under temperate latitudes, testicular characteristics and testosterone blood levels were better during the third than during the second year of age in breeds of sheep under temperate latitudes Mandiki et al. (1998a,b). Based on these evidences, this paper attempts to test under a Mediterranean environment, whether treatment with melatonin improves reproductive output of growing rams. Other studies, using adult rams that have already been exposed to natural increased photoperiod in spring (Rosa et al. 2000; Palacin et al. 2008) have proved the efficiency of exogenous melatonin to improve reproduction. Development of a method to improve reproductive efficiency of younger males may be interesting in reducing production costs, accelerating benefits of genetic selection, and allowing earlier progeny testing. The specific aim

Correspondence: Mourad Rekik, Diversification and Sustainable Intensification of Production Systems, International Centre for Agricultural Research in the Dry Areas (ICARDA), P.O. Box, 950764 Amman 11195, Jordan. (Email: m.rekik@cgiar.org)

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of this paper is therefore to investigate if a treatment with melatonin subcutaneous implants – during winter season when photoperiod is increasing – affects testicular traits, plasma testosterone concentrations and mating capacity of young rams of the Barbarine breed.

MATERIALS AND METHODS

The study was conducted on the pilot farm of the Pasture and Livestock Agency (OEP Tunisia) located in Souel (Central Tunisia, latitude 35° North). This location has a Mediterranean-type climate, cool winters and hot dry summers and an average annual rainfall lower than 300 mm. The experiment conforms to national ethics guidelines for animal usage in research.

Animal treatments and experimental procedures

In late February, 14 young rams were randomly obtained from a flock of improved rams and were used in this study. The animals were all aged 16 months and were raised from a flock of young improved rams and were used in this study. The mean ± SE body weight was 55 ± 4.3 kg, while the mean scrotal circumference, measured on 25 February at the widest part of the scrotum, was 26.7 ± 1.90 cm. All the animals were checked for integrity of their sexual organs prior to the experiment, and they were vaccinated against enterotoxemia and received oxfendazole (5 mg/kg body weight) against internal parasites.

Seven of the 14 rams received each, three melatonin implants on February 28 (Melatonin group), while the remaining seven rams (Control) went untreated. Melatonin was administered in the form of a subcutaneous implant, Melovine® (CEVA – Animal Health, Tunis, Tunisia) placed under the skin, near the base of the ear with a Melovine applicator gun. Throughout the experimental period, rams were allowed to graze available vegetation cover of native Medicago spp. They were daily supplemented with 0.7 kg/ram of a soybean meal barley-based concentrate.

Data collection

Measurement of live weight and scrotal circumference

Live weight and scrotal circumference were measured at 2-week intervals. At each occasion, the rams were weighed twice before being fed in the morning. Scrotal diameter was assessed with a measuring tape (± 1 mm) at the maximum antero-posterior level of the scrotum with the ram in a standing position. No correction was made for scrotal skin thickness.

Semen collection and evaluation

Starting 3 weeks after treatment with Melovine implants, volume and sperm concentration of the ejaculates were measured at weekly intervals by collecting semen using an artificial vagina. This was performed by the same operator and ejaculates were immediately kept in a water bath at 35°C. At each occasion, the rams in Melatonin and Control groups were put individually in the collection room in presence of a teaser female that was previously induced into estrus. Estrus was induced by inserting a progestagen-impregnated vaginal sponge for 6–7 days followed by daily injections of 250 μg of estradiol benzoate for three consecutive days (Baril et al. 1993). Ejaculates are recovered in graduated glass tubes (4 mL) graduated to the nearest 0.1 mL. This allowed direct determination of the ejaculate volume without taking into account the frothy part on the top. Concentration (number of spermatozoa/mL) was determined using a spectrophotometer that is calibrated to measure sheep sperm concentration at 550 nm (Accucell®, IMV, Paris, France). Four microliters of fresh semen were diluted in 3996 μL of physiological saline solution. Mass activity (wave motion or motility score) in undiluted semen was assessed by examining a drop of semen under a warm stage using a phase contrast microscope at ×100 magnification (score, 0–5).

Mating behavior (libido)

Eight weeks after the animals were implanted with melatonin, rams were ordered in a random sequence within treatment and exposed to two ewes in estrus for a 15-min period in a test pen of 6 m². The test facility was constructed to eliminate outside distractions and to prohibit the ram being tested from seeing any sheep other than the teaser ewes. Libido was assessed by observing displays of sexual behavior and recording the number and type of responses each ram exhibited toward the ewes. Non-mating sexual approaches included vulva sniffing, Flehmen, lateral approaches and mount attempts. Period to first estrus reaction and total activity time were also recorded.

Plasma testosterone concentration

At approximately 60 days after the implantation and for six consecutive hours, serial blood samples were taken every 20 min from the jugular vein of all the rams using heparinized vacutainers. After centrifugation (3000 x g, 15 min, 4°C), plasma was aspirated and stored at −20°C until assayed for plasma testosterone concentrations. A testosterone pulse was defined as an increase in hormonal levels followed by a decrease and when the amplitude of the increase is greater than the mean of all samples taken in the 6-h period plus two standard deviations (Diekman et al. 1991).

Testosterone assay procedures

The concentrations of testosterone in plasma were determined using a single antibody, non-extraction radioimmunoassay method. Plasma testosterone concentrations were determined in duplicate using a Coat-A-Count® radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA, USA), according to the manufacturer’s instructions. The limit of detection was 0.02 ng/mL. Inter- and intra-assay variation coefficients were 4.4% and 8.2%, respectively.

Statistical analysis

Results relative to live weight, scrotal circumference, semen volume, sperm concentration and mass activity of the ejaculates as well as plasma testosterone concentrations were analyzed using the MIXED models procedure (SAS Version 9.1; SAS Inst. Inc., Cary, NC, USA). Sources of variation included treatment with melatonin and measurement time. The random variable was ram within treatment. Data on the number of testosterone pulses, mean testosterone concentrations and mating behavior traits were compared using
analysis of variance (ANOVA) (SAS 2005) with melatonin treatment as the main effect. Testosterone pulse frequency was converted to the square root of \( n + 0.5 \) because some rams, particularly in the Control group, had zero pulse frequencies (Dickson & Sanford 2005). Whenever significance was reached, mean treatment groups were compared according to the null-hypothesis by the least-square mean option (SAS 2005). The effect of treatment was considered significant when the level of probability was 0.05 or less. Trends for the treatment to affect measured traits were admitted for levels of probability comprised between 0.05 and 0.1. Correlations (Pearson correlation) between libido and testosterone traits were measured. Results are presented as mean ± SEM.

RESULTS

Live weight and scrotal circumference

Throughout the experimental phase, live weight of rams in both treatment groups increased \( (P < 0.05) \). For both treatment groups, average initial live weight was 54.6 ± 4.32 kg. After 60 days of implantation, live weight of Melatonin and Control rams reached 62 and 61 kg \( (\text{SEM} = 3.8) \) respectively \( (P > 0.05) \). During the 60-day experimental period and regardless of treatment, scrotal circumference increased from an initial value of 26.7 ± 1.90 cm to a final average value of 30.8 ± 1.81 cm \( (P < 0.05) \). For Control and Melatonin rams, initial values were 26.7 ± 1.79 and 26.7 ± 2.15 cm, respectively. The difference between treatment groups was more pronounced at 60 days when scrotal circumference reached average values of 32.1 ± 1.54 and 29.5 ± 1.0 cm for Melatonin and Control animals, respectively \( (P < 0.02) \).

Semen characteristics

Semen was collected easily from all rams. Semen characteristics, such as ejaculation volume, sperm concentration and mass activity score are depicted in Figure 1 for animals in both treatments. Regardless of the treatment, mean ejaculation volume was 0.96 ± 0.301 mL \( (0.93 ± 0.208 \text{ and } 0.98 ± 0.534 \text{ for Control and Melatonin rams, respectively}) \), mean sperm concentration was 5.07 ± 0.971 \( \times 10^9 \) spermatozoa/mL \( (5.10 ± 1.162 \text{ and } 5.05 ± 0.813 \text{ for Control and Melatonin rams, respectively}) \) and mean mass activity score was 4.08 ± 0.575 \( (4.00 ± 0.531 \text{ and } 4.19 ± 0.456 \text{ for Control and Melatonin rams, respectively}) \). Melatonin treatment tended \( (P = 0.091) \) to increase sperm concentration 60 days after implantation. At this time, average values reached 5.87 ± 0.703 and 4.61 ± 0.654 \( \times 10^9 \) spermatozoa/mL for rams in the Melatonin and Control groups, respectively.

Mating behavior

Total activity time and number of lateral approaches increased \( (P < 0.001) \) as a result of melatonin treatment (Table 1). Furthermore, melatonin-treated rams attempted more mounts \( (P < 0.01) \) than control counterparts (Table 1). Four rams of the Control group did not attempt any mount. Other measured sexual behavior traits were not affected by melatonin administration.

<table>
<thead>
<tr>
<th>Latency to first reaction (s)</th>
<th>Experimental group</th>
<th>Prob. &gt; F</th>
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<tr>
<td>Melatonin</td>
<td>1.8 ± 0.83</td>
<td>1.5 ± 0.57</td>
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<td>Control</td>
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<th>Total activity time (min)</th>
<th>Experimental group</th>
<th>Prob. &gt; F</th>
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<tr>
<td>Melatonin</td>
<td>9.8 ± 0.44</td>
<td>5 ± 1.41</td>
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<td>Control</td>
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<th>Experimental group</th>
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<td>Melatonin</td>
<td>11.6 ± 5.77</td>
<td>8.6 ± 3.78</td>
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<th>Experimental group</th>
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<tr>
<td>Melatonin</td>
<td>3 ± 1.58</td>
<td>1.8 ± 1.64</td>
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<td>Melatonin</td>
<td>34.2 ± 9.64</td>
<td>14.8 ± 11.07</td>
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<td>Control</td>
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<tr>
<th>Mount attempts</th>
<th>Experimental group</th>
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<tr>
<td>Melatonin</td>
<td>1.8 ± 0.44</td>
<td>0.2 ± 0.44</td>
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<tr>
<td>Control</td>
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Plasma testosterone levels

Plasma testosterone concentrations for the two treatment groups, during the 6-h sampling period, are shown in Figure 2. In both groups, plasma testosterone concentrations showed a sustained increase from initial values of 1.21 and 0.49 ng/mL for Melatonin and Control individuals, respectively. They reached a peak at approximately 200 min (24.31 and 12.24 ng/mL for Melatonin and Control rams, respectively); then started to decline steadily until the end of the sampling period ($P < 0.01$). All along the first 14 samplings, plasma testosterone concentrations were higher ($P < 0.05$) or tended ($P = 0.067$ for the eighth sampling at 140 min) to be higher for melatonin-treated rams in comparison to their Control counterparts. Thereafter, testosterone concentrations were similar. Both mean plasma testosterone concentrations (9.4 ± 6.45 vs. 4.40 ± 4.15 ng/mL; $P < 0.001$) and testosterone pulse frequency (3.4 ± 2.24 vs. 1.2 ± 1.0; $P = 0.086$) increased as a result of melatonin treatment. For each of the treatments and for three different rams, profiles of testosterone variations during the 6-h sampling period are shown in Figure 3. For Melatonin-treated rams, six pulses occurred in comparison to only two in the Control animals. For these individual profiles, areas delineated by measured concentrations were calculated using the trapezoid rule and average values were 1620.3 and 3503.8 ng/mL (SEM = 452.81) for Control and Melatonin rams, respectively ($P < 0.001$). In Melatonin-treated rams, there was a trend ($P = 0.07$) for a significant correlation between total activity time and the highest concentration of testosterone reached over the 6-h sampling period ($r = 0.833$).

DISCUSSION

Unlike puberty which is well documented in rams (Setchell 1993), the transition period from puberty to sexual maturity is much less well characterized. Postpubertal rams have a low fertility as a result of poor semen quality (Colas & Zinszner 1975) and their libido is inferior to older animals (Dickson & Sanford 2005). It was clearly demonstrated in the present study that young adult rams of Barbarine breed (latitude 35° North, semi-arid environment) which received melatonin treatment presented increased scrotal circumference, improved libido and elevated testosterone concentration. Some features of these findings are important to consider.

First, it has been established that rams need to have experienced a previous period of long days (1.5–2 months duration) in order to respond to inductive short days or alternatively to melatonin treatment (Chemineau et al. 1996). In our study, the young rams received the implants late February when the light-to-dark ratio is much shorter than the 16 h light : 8 h dark commonly standing for long days (Rosa et al. 2012). Therefore, for a reputed low seasonal breed (see review paper by Ben Salem et al. 2011), it may be speculated that reproduction of young rams of this breed is constrained by narrow variations in photoperiod between the winter solstice and late February. Furthermore, Martin et al. (1994) reported that photoperiod may be involved in the onset of reproductive

Figure 2. Mean (± SEM) plasma testosterone concentrations over a 6-h sampling period of control and melatonin-treated young rams (**, *, NS: per sampling time, differences between treatments groups are highly significant ($P < 0.01$), significant ($P < 0.05$) or non-significant respectively).
period of Merino rams when reared in a Mediterranean-type environment. Similar to our results, melatonin treatment as early as immediately after the winter solstice (Forcada et al. 2002) or early in January–February (Abecia et al. 2007) was shown to be effective for Spanish sheep breeds.

Second, scrotal circumference and testosterone secretion increases in melatonin-treated rams are not related to body weight as growth patterns were similar in both groups. Similar findings, as concerns scrotal circumference, testosterone secretion and body weight, were reported for Corriedale rams (Pérez-Clariget et al. 1998). Furthermore, the improved mating behavior in melatonin-treated rams could be explained by the increased testosterone concentration and pulse frequency. This is partly confirmed by the positive correlation between total activity time and the highest concentration of testosterone reached over the 6-h sampling period in melatonin-treated rams. It is also interesting to note that melatonin-treated rams...
were exposed to much higher levels of circulating testosterone throughout the day (data of the area delineating the profiles), further explaining their increased libido.

Third, melatonin treatment increases total activity time and numbers of lateral approaches and mount attempts. These findings have direct, practical implications on the outcome of the flocks’ reproductive performances when yearling rams are put to reproduction for the first time. This is also very important in a production system where out-of-season breeding is very common (Rekik et al. 2005). Similar results were obtained by Rosa et al. (2000), when melatonin treatment was initiated in mid-May (end of spring), when the animals had already been naturally exposed to months of increasing day length. However, in the northern hemisphere, the application of melatonin in mid-March (spring), without the priming period of long days, has not resulted in a positive response regarding sexual behaviour, testis diameter, semen volume and sperm concentration (Williams et al. 1990).

Finally, neither measurement time nor treatment presented an effect on semen characteristics. Marked inter-individual variations may prevent changes in semen characteristics, although melatonin-treated rams tended to present increased sperm concentration at the end of the experimental period. In melatonin-treated rams, scrotal circumference increase and mostly mating behavior improvement are satisfactory. Although it was not part of this study, it has been recently shown that administration of melatonin during anoestrus to rams can improve fertility rate by increasing sperm progressive motility from days 45 to 90 after administration (Casao et al. 2010).

Conclusion
The abrupt application of melatonin without a priming period of long day length, recommended by Hanif and Williams (1991), provides a significant advantage regardless of management difficulties and financial costs of photoperiod treatment. Therefore, the early melatonin treatment of young rams from breeds thriving in a southern Mediterranean region could provide a less costly management practice to manifest their reproductive potential.

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