Melatonin modifies scrotal circumference but not plasma testosterone concentrations and semen quality of rams during the seasonal anestrus at 43°S

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Short title: Melatonin treatment in rams

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Treatment with exogenous melatonin has become one of the hormonal treatments widely used to induce sexual activity in rams and ewes during the seasonal anoestrous. This paper describes the effect of melatonin implants on semen characteristic, scrotal circumference and plasma testosterone levels of rams in the Patagonia region of Argentina. Results indicate that whereas scrotal circumference enlarges in a great extent after melatonin treatment, semen quality parameters are not modified.

Abstract. The effect of melatonin implants on semen quality in fresh and frozen/thawed semen, Scrotal circumference and plasma testosterone levels has been studied During two consecutive years in the Patagonia region of Argentina (43ºS). Sixteen Dohne Merino rams (experiment 1) and 12 Dohne Merino and Merino rams (experiment 2) were used from September to December 2011 and 2012, respectively. Half of the rams were treated with melatonin implants (M groups), the remaining rams being the non-treated, control group (C groups). Differences between groups ($P<0.01$) for scrotal circumference were observed from 30 days after melatonin implantations up to the end of experiment 1, M rams presenting a higher scrotal circumference than animals in the C group. In the second experiment, similar differences between groups ($P<0.01$) were observed from day 45 to day 75. No differences between groups were detected for any of seminal parameters under study (volume, sperm concentration, mass motility and percentage of live sperm) and plasma testosterone levels. Semen quality of frozen/thawed semen was similar between treated and non-treated rams (rectilinear progressive individual motility, percentage of live spermatozoa, and membrane permeability test). No significant differences for the parameter derived (mitochondrial functionality) and the chlortetracycline (acrosomal state) tests were observed. In conclusion, the use of melatonin implants during the seasonal anoestrous at 43ºS provokes a significant increment of the scrotal circumference of rams without changing seminal quality parameters of fresh and frozen/ thawed semen.

Additional Keywords: photoperiod, testosterone, Southern hemisphere

Introduction

Argentina's Patagonia region is characterized by its environmental variability, located between 40º N, 52º S, 65ºE, 73ºW with a regimel rainfall averaging 130 mm and temperatures of 30ºC in summer and in winter -5. The drought, one of the main environmental problems of this area, not allow livestock development. Several decades ago, due to wool international market conditions and sheep adaptability to such environmental conditions favored the development of flock whose main purpose was the production of wool. The loss of these markets brought about a major economic and social loss in the area. Using a dual purpose breed as Dohne Merino, has been a good alternative to improve production rates that maintain the quality of merino wool with good meat productionis one of the major environmental problems is punishing extensive livestock systems in recent years, with important implications productive basically cattle stock decreased by limiting the contribution nutritional fields, being this area purely wool production. The use of a dual purpose as Dohne Merino breed,has been a good alternative to improve indexes of production maintaining the finesse wool of a merino and incrisingproduction.Reproductive seasonality is a limiting factor for the annual lamb production in sheep farms.

.Photoperiod is responsible of this limitation, through the circadian secretion of melatonin by the pineal gland, which conveys to the neuro-endocrine system information about photoperiod (Bittman et al., 1983). The administration of subcutaneous melatonin implants during the non-reproductive season reverses the reproductive effect of seasonality in rams and ewes (Haresign et al., 1990).

in rams, The use of melatonin implants has revealed numerous effects on their reproductive physiology, such as increase in scrotal diameter, improvement of sperm quality (Casao et al., 2010a) and improvement in the benefits of some artificial insemination centers (Beltran de Heredia, 1995); it also seems to increase libido (Bravo and Roy, 2003) and reproductive performances of those ewes mated by implanted rams (Palacin et al., 2008). Moreover, the treatment of rams with melatonin between the middle and the end of spring accelerated the seasonal increase in LH levels and testis size (Webster et al., 1991) and increased the proportion of ewes that ovulated in response to the introduction of a ram (Rosa et al., 2000). It has been demonstrated melatonin seasonal variations in ram seminal plasma, and its correlation with seminal plasma testosterone levels and antioxidant enzyme activity (Casao et al., 2010b). More recently, and for the first time, it has been evidenced the presence and distribution of melatonin receptors (MT1 and MT2) in ram sperm cells (Casao et al., 2012).

To our knowledge there is no information about the effect of melatonin implants on semen characteristics in latitudes above 40° in the Southern hemisphere. For instance, in New Zealand, the effect of melatonin implants on the early season on performance of Romney and Poll Dorset rams was investigated over three consecutive years (Muir et al., 1993).

The aim of this study was to determine the effect of melatonin implants on quality parameters of fresh and frozen/thawed semen, scrotal circumference and plasma testosterone levels in Dohne Merino and Merino rams, at 43ºS.

Material and methods

The experiments were conducted at the Laboratory of Animal Reproduction of the INTA Research Station (Trelew, Chubut; 43ºS 65ºW). These facilities have been approved by SENASA (National Health Service and Food of Argentina). All procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of experimental animals.

Animals and experimental procedures

Adults dohne Merino and Merino rams were used. Between September and December (non-reproductive season, South Hemisphere) scrotal circumferences, plasmatic testosterone level and quality parameters in fresh and frozen/thawed sperm were evaluated. The assessments were done twice a month, with an intervals of 15 days. The experiment was repeated in the next year. In both experiments, rams were kept outdoors and fed with alfalfa hay and corn, to provide their maintenance requirements (AFRC, 1993), with unrestricted access to water. - Subcutaneous Implants contained 18 mg de melatonin (Melovine, CEVA Salud Animal, Barcelona, Spain) were inserted by a special syringed in rams ear.In the first year of study (experiment 1) . Sixteen Dohne Merino rams, with a mean liveweight (LV) of 94.5±0.9 kg and a body condition score (BCS) of 3.03±0.01 (Russel et al., 1969) were used . Animals were divided into two groups. Group M (n=8) was conformed by rams treated with 3 subcutaneous melatonin implants on September 3th (day 0) and group C (n=8) was conformed by non-treated rams (control).

In the second year of study (experiment 2), Twelve rams (LW: 92.5 kg; BCS: 2.74±0.08) were used. Animals were divided into two groups: Group M (n=6; 3 Dohne Merino and 3 Merino) and Group C (n=6; 3 Dohne Merino and 3 Merino).

and fed alfalfa hay and corn, to provide their maintenance requirements (AFRC, 1993), with unrestricted access to water.

Samples Collection

Scrotal circumference and blood samples were taken fortnightly from melatonin implantation (day 0) until the end of the experiments. Semen was collected from 30 days of melatonin implant placement, during 8-week in experiment 1 and 10 weeks in experiments 2, between 8:30 and 9:00 a.m. using and an artificial vagina in 3-ml graduated tubes . Collected samples were placed in a bath at 37°C and volume, sperm concentration, mass motility and percentage live sperm were recorded.

Freezing and thawing protocol

Between days 60 and 70 of the experiment, semen was frozen to evaluate semen quality parameters after thawing.

Between days 60 and 70, semen was frozen to evaluate semen quality parameters after thawing. Semen was extended in Triladyl (250 g.,Minitub, Germany) to a final concentration of 100 x 10^6 sperm/ml and packed in 0.25 ml straws. The diluted samples were slowly cooled to 6°C (3º/2 min), kept in a fridge for 24 h at 6ºC and frozen with nitrogen vapor for 2 min at 20 cm and 3 min at 9 cm, and finally stored in liquid nitrogen (-196ºC). Thawing was carried out by

dropping the straws in a water bath at 37ºC for 1 min and pouring the semen in dry tubes and incubated for 5 min at the same temperature.

were keep in shed outside and fed with a diet of alfalfa and grain maize.

9 *Frozen*/thawed *Semen quality Assessment*

10 Volume (ml) was calculated by direct observation of collection with 0.1 ml precision and sperm concentration (sperm/ml) was calculated by the use of the Neubauer chamber.

The evaluation of sperm movement wave (mass motility) was conducted using a subjective scale of 0 to 5 (0: no moving sperm and5: extremely high waves, >90 % mobile sperm). For this determination, a 6 µl aliquot of the ejaculate was placed onto a slide on a hot plate at 37°C at 40 x magnification and the same trained technician performed the evaluation throughout the study. Assessed visually under a 40x),(Nikon Diaphot, Japan).Total sperm and rectilinear progressive individual motilities involve visualizing and subjectively estimating the proportion (%) of mobile sperm or mobile sperm with forward displacement, respectively, by observing a 6 Ml aliquot in at least two microscopic fields using an optical microscope (400 x) at 37°C.

The percentage of sperm cells with intact plasma membranes was measured according to Mortimer (1994) with modifications. Briefly, semen samples (6 ll) were mixed during 10 s with an eosin–nigrosine solution and then smeared onto a pre-warmed microscope slide and air-dried. At least 200 cells were evaluated per slide using light microscopy (4009). Pink-coloured sperm were considered as sperm with a damaged plasma membrane, and unstained cells were recorded as sperm with intact plasma membrane.Total sperm and rectilinear progressive individual motilities involve visualizing and subjectively estimating the proportion (%) of mobile sperm or mobile sperm with forward displacement, respectively, by observing a 6 Ml aliquot in at least two microscopic fields using an optical microscope (400 x) at 37°C.

1 The functionality of sperm plasma membrane was evaluated using the hypo-osmotic swelling test (HOS) (Jeyendran et al. 1984; Garcıa Artiga 1994). A volume of 10 ll of semen was added to 1 ml of the hypoosmotic solution (100 mOsm/l, 57.6 mM fructose and 19.2 mM sodium citrate) and incubated at 37°C for 30 min. After incubation, one drop of semen was placed on a glass slide, covered with a cover slip and evaluated under a phase-contrast microscope (4009). At least 200 sperm were counted, and the proportion of sperm with swollen or coiled tail was calculated (HOS+).The hypoosmotic sweeling test (HOS test) has been used to study the functionality of the flagellar plasma membrane compartment, based on the method described by Jeyedran et al (1984) in the human species, adapted to the study of sheep semen by Garcia-Artiga (1992).

Mitochondrial functionality was evaluated by the specific probe rodhamine 123 (Rh 123), a cationic lipophilic fluorochrome that accumulates selectively inside of active mitochondria, coupled with propidium iodide (PI) stain to discriminate between living and dead sperm (Evenson et al. 1982). An aliquot of 250 µL of semen sample containing 6 x109 cells was mixed with 1 mL of isotonic solution (140 mM ClNa; 10 mM glucose; 2.5 mM ClK; 20 mM Hepes; 0.5 mM polyvinyl alcohol; 0.5 mM polyvinylpyrrolidone) and 3 µL of Rh 123 (0.2 mM) and

incubated at 37 ºC for 30 min in the dark. After the addition of 25 µL PI solution (0.5 mM), the samples were incubated for 15 min and the reaction was stopped by the addition of 10 µL of a formalin isotonic solution. Cells were examined under a Nikon fluorescence microscope at 546 nm and four subpopulations of cells were observed: cells with functional mitochondria with an intact (Rh+/PI-) or damaged plasma membrane (Rh+/PI+), and cells without functional mitochondria with an intact (Rh-/PI-) or damaged (Rh-/PI+) plasma membrane. At least 200 sperm were counted and the proportion of sperm with functional mitochondria and intact plasma membrane was calculated.

IN VITRO capacitation status was assessed with the chlortetracycline fluorescence assay or CTC staining as described by Pérez et al. (1996), with modifications (Gil et al., 2003). A CTC solution (750 µM) was freshly prepared in a buffer containing 20 mM TRIS,130 mM NaCL, and 5 mM DL-Cysteine and pH 7.8. A 5 µL of semen was mixed with 20 µL of CTC-working solution. After 20 s, the reaction was stopped by the addition of 5 µL of 1% (v/v) glutaraldehyde in 1 M Tris-HCL pH 7.8. Smears were prepared in a clean microscope slide, covered with a coverslip, sealed with colorless nail varnish and stored in the dark at 4 °C. Samples were examined under an epifluorescent inverted Eclipse TE-300 microscope (Nikon, Japan) within 12 h after staining, using green filters (380 nm excitation and emission at 420 nm). At least 200 stained sperm were classified into three categories, according to their CTC-staining patterns (Fraser et al., 1995) as follows: F pattern (uniform bright fluorescence in the head with a fluorescence band in the equatorial segment; non-capacitated sperm with an intact acrosome); B pattern (fluorescence-free band in the post-acrosomal region of the head; capacitated sperm with an intact acrosome) and AR pattern (uniform bright fluorescence over the entire sperm head and full fluorescence along the equatorial segment; sperm that hadundergone an acrosome reaction).

2

3 *Testosterone analysis*

4 Plasma samples were taken to every 14 days from 30 days of melatonin implant placement, the same as Kokolis et al (2000) to determinate plasma testosterone concentrations analysed by a single radioimmunoassay (RIA). The minimum detectable concentration was 0.02 ng/ml. Intra-assay CV was 8.3%.

5 *Statistical analyses*

1 All variables were subjected to analysis of variance using the mixed procedure of SAS (v. 9.0, Statistical Analysis System Institute Inc., Cary, North Carolina, USA) with repeated measures data. Since no differences between breeds and years were observed, these factors were not considered into the model. Frozen/thawed semen characteristics were analyzed by ANOVA. Means were considered different when $P<0.05$, and tendency to differ when $P\leq0.10$.

2

3 *Results*

Treated and non-treated rams presented an increase in scrotal circumference gradually throughout both experiments. During the first trial, differences between melatonin implantations) up to the end of the experiment, M rams presenting a higher scrotal

circumference than C animals. In the second experiment, differences between groups ($P<0.01$) were observed from day 45 to day 75 after the onset of melatonin insertions (Fig. 1).

4 The evolution of mean ejaculate volume, sperm concentration, mass motility and percentage of live sperm throughout both experiments are presented in Figures 2 to 5, respectively. No differences between groups were detected for any of the parameters under study.

5 In experiment 1, a fast increase of plasma testosterone levels during the first 45 days after melatonin implantation was observed, followed by an irregular pattern of rising and falling throughout the experiment, with no significant differences between treatments at any time (Fig 6). Testosterone levels during the second experiment showed a gradual increase throughout the studied period in the group of animals implanted with melatonin, being irregular for the control group, with a maximum value four months after the onset of the treatment; again, no differences between groups were detected.

6 Table 1 shows the results of the analysis of semen quality of frozen/thawed semen.

No significant differences between treatments were observed for rectilinear progressive individual motility, mass motility, percentage of live spermatozoa, and membrane permeability test (HOS test). Significant differences ($P<0.05$) in the overall assessment of sperm motility was observed in the first experiment, the control group presenting a higher percentage than the treated one. This difference was not detected in the second year. No significant differences for the rest of the parameters derived from the Rhodamine and the Chlortetracycline tests were observed.

1 *Discussion*

2 In general, semen quality has not been affected by the exogenous melatonin administration, so treatment did not affect semen volume or sperm concentration during the collection period. These observations were consistent and similar in the two years under study. It is likely that melatonin administration initiated in spring, without a priming period of long days preceding the treatment, is not sufficient stimulus to increase sperm production, as has been described by Rosa et al. (2012). Moreover, in a study carried out in Hungary (Faigl et al., 2009) no differences were found in concentration of spermatozoa, total motility, and number of spermatozoa with fast and slow progressive motility and normal/abnormal morphology between the melatonin-treated rams and control group, although, in melatonin-treated animals, basal and GnRH-induced testosterone levels were slightly elevated on day 47 and became significantly higher on day 71 as compared to controls. Bravo and Roy (2003), who used melatonin implants in Ile de France, Merino Precoce and Fleischschaf rams, found no statistical differences on ejaculate volume, only sperm concentration for those animals implanted with melatonin was statistically significant. Beltrán de Heredia (1995), using Laxta rams, found a significant improvement of volume and sperm concentration with the use of melatonin from the ninth week after the implant was placed, although no differences in the rest of sperm quality parameters were found. Kaya et al. (2000) observed that melatonin implants during the breeding season did not affect semen quality parameters, but recorded a significant improvement on sperm morphology and motility when administered outside the breeding season.

3 The increment of scrotal circumference experimented by all males, treated and non-treated was in agreement with that observed previously in Mediterranean breed, an increase in testicular size from spring to summer (Avdi et al, 2004; Santiago-Moreno et al., 2005). In

particular, Palacín et al. (2008) obtained statistical differences in favour of treatment with melatonin in the scrotal circumference of Assaf, Rasa Aragonesa and Manchega rams.

The evolution of plasma testosterone levels throughout both experiments was very irregular and, in spite of the higher levels in the treated group, no significant differences between groups were observed. However, it is likely that this non- significant difference in testosterone was sufficient to enlarge scrotal circumference in the treated group. Kaya et al. (2000) obtained statistical differences in plasma testosterone levels of Merino rams out of the breeding season from 70 days of implant insertions, and found no differences during the breeding season. The results of our experiments also differ from those obtained by Casao et al. (2013), who observed significant differences in favor of the treated group after the fourth week of implantation in Rasa Aragonesa rams, expressing their maximum levels of testosterone twelve weeks after the onset of melatonin treatment. The action of melatonin on testosterone levels in both blood and seminal plasma could be due to the stimulating effect of the pineal hormone in the hypothalamic-pituitary axis resulting in the increased level of testosterone through increased secretion of GnRH and LH (Misztal et al., 2002).

Regarding the results of the evaluation of frozen/thawed semen from both trials, only a significant difference on total sperm motility for the control group in the first experiment was found; the remaining variables did not generate statistically differences. Casao et al. (2007) observed that melatonin treatment during seasonal anoestrous in Rasa Aragonesa rams increased the percentage of progressive motile sperm, although other authors found no significant difference for this parameter (Kaya et al., 2000).

In the case of assessing the status of capacitation with CTC, Gillan and Maxwell (1999) indicated that dilution, freezing and thawing of semen induce structural changes that lead to a premature sperm capacitation. However, it is possible that dead cells lose their acrosomal contents due to the rupture of the membrane, presenting a similarity with the acrosomal changes observed during capacitation (Marti et al., 2000). Therefore, it is necessary to assess the state of capacitation only in those sperm that survive freezing.

Conclusion

The use of melatonin implants during the seasonal anoestrous in Dohne Merino and Merino rams in the Patagonia region of Argentina, at 43ºS, provokes a significant increment of the scrotal circumference without changing seminal quality parameters of fresh and frozen/ thawed semen.

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