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Seminal Profile, Antioxidant Enzymes Activities and Levels of Testosterone in Tyrolean Mountain Rams after Melatonin Implantation

[1,2]El-Battawy, K. A. and [2]Sckalcki, M.

[1]Animal Reproduction and Artificial Insemination Dept., National Research Centre, Egypt.
[2]Physiology, Pathophysiology and Experimental Endocrinology Dept.,University of Veterinary Medicine, Austria.

ABSTRACT

This investigation was conducted to study seminal profile, antioxidant enzymes activities and testosterone levels of Tyrolean mountain rams during non-breeding season. Five rams were implanted with melatonin, and other five were used as a control group. Exogenous melatonin treatment significantly ($P < 0.05$) increased ejaculate volume motility Score**,** sperm concentration, alive sperm %, total sperm output and abnormalities % . Regarding, the antioxidant enzymes activities, melatonin significantly decreased ($P < 0.05$) malondialdehyde concentration and increased significantly ($P < 0.05$) glutathione peroxidase, superoxide dismutase and catalase activities. Further, melatonin implants increased significantly ($P < 0.05$) testosterone levels. In conclusion, melatonin implantation improved semen quality, antioxidant enzymes activities and increased testosterone concentration during the non-breeding season in Tyrolean mountain rams.

Key words: Melatonin, semen; Tyrolean; antioxidant; testosterone

Introduction

Sheep living in the temperate climate zone are seasonal breeders and that seasonality occurs in rams but it is less marked than in ewes because spermatogenesis and sexual activity didn't stop (Casao *et al.,* 2010a). Melatonin (*N*-acetyl-5-methoxytryptamine) is an indoleamine secreted from the pineal gland and it is the key hormone in interpreting photoperiods to organize seasonal changes (Ebling and Hastings, 1992). It plays crucial roles in several fields of physiology, e.g. in the nervous system, antioxidant defence mechanism, immune system and gastrointestinal tract, as has been reported in recent years (Reiter *et al.,* 2009; Hardeland *et al.,* 2011; Carpentieri *et al.,* 2012). Under normal seasonal fluctuation of daylength rams exhibit significant variation in their reproductive performance.

Melatonin administration in ewes has been recorded to advance the onset of reproductive activity (Abecia *et al.,* 2006; Papachristoforou *et al.,* 2007). Moreover, melatonin has been reported to exert beneficial effects on ovulation and conception rates in ewes (El Battawy, 2006; deNicolo *et al.,* 2008; Tsiligianni *et al.,* 2009).

On the other hand, in young rams under a long daylight melatonin administration increased gonadotropin and testosterone concentrations and probably regulates antioxidant enzyme activity (Kokolis at al., 2000) while in mature rams, melatonin implants improved testicular growth and semen characteristics in different breeds during out of season breeding (Kaya *et al.,* 2000; Casao *et al.,* 2010b).

Melatonin treatment during the non-reproductive season, mainly by means of subcutaneous melatonin implants, seems to reverse the reproductive effect of seasonality in both rams and ewes (Haresign *et al.,* 1990). GardeLópez-Brea *et al.,* (1996) reported that melatonin implants had positive impact on seminal parameters when the implant was administered on 17th May.

Tyrolean mountain sheep breed is an endangered breed result from cross-breeding between the Austrian Tiroler Steinschaf of the Tyrol with the Italian Bergamasca breed from the area of Bergamo. It is distinguished by high climatic tolerance and high daily weight gains, (Kareta *et al.,* 2000).

Thus, the aim of this study was to determine the variation of testosterone levels and semen parameters after melatonin treatment of rams during the non-reproductive season.

Materials and Methods

The current study was conducted at the Experimental Farm of the University of Veterinary Medicine (Schafstall) in Berndorf, Austria during the non-breeding season (from 17 May to 17 July)

Corresponding Author: El-Battawy, K. A., Dept. Animal Reproduction and Artificial Insemination, National Research Center, Egypt.
E-mail: ekhairi@hotmail.com

Animals

Ten Tyrolean mountain rams (age: 24–34 months) were included in the trial. The animals were housed in groups and fed alfalfa hay and concentrates. They were turned out to pasture daily. The animals had free access to fresh water and mineralized salt blocks. The rams were divided into two equal groups. The melatonin group (M) was implanted subcutaneously twice with a single slow-release melatonin capsule (18 mg melatonin implant; Melovine®, Ceva Santé Animale,, Libourne, France) first at the beginning of the trial (d 0) and then 30 days (d 30) later. The implant was given subcutaneously high on the neck, near the base of the ear using special gun. The control group (C) received no implant.

The following parameters were measured in all experimental animals during the 63-day trial.

The rams had previously been trained for semen collection using an artificial vagina (Minitüb, Tiefenbach, Germany). Semen was collected twice weekly by means of an artificial vagina adjusted at 42ºC. After collecting the semen, the ejaculates were transferred to the laboratory within 30 seconds and kept in a water bath for 10 min at 37°C for preliminary evaluation of sperm motility and live percentages by means of conventional methods. Only ejaculates >70% initial motility and 60 million motile sperm cells /mL were used in the following experiments after being pooled to avoid the ram influence: Semen characteristics were studied as follows:

Semen samples were collected into graduated tubes that directly connected to the artificial vagina where semen volume was immediately measured using pipettes and prepared for further investigations. Total sperm output (10^9/ejaculate) was calculated by multiplying semen ejaculate volume by semen concentration. A drop of raw, undiluted semen was put on a glass slide heated to 37ºC and mass motility was classified as described by Evans and Maxwell (1987) between grades 1 and 5 under low-power magnification (100x) using a warm plate light microscope. Sperm concentration was assayed according to Smith and Mayer (1955). A weak aqueous eosin solution to kill sperm cells and stain the head purple color. Counting of sperms was done by Thomas ruling double counting improved Neubauer hemocytometer slide (GmbH + Co., Hamburg, Germany).

Live sperm (%) were estimated according to Blom (1977). Stained smear was prepared directly after ejaculation using an eosin-nigrosine staining mixture at 1:4 dilution rate. Two hundred spermatozoa per sample were examined for morphology and viability in stained smear at 1000x magnification (oil immersion). The principle of these techniques is dye exclusion as red eosin stains dead sperm head while nigrosine provides a blue-black background. For the evaluation of sperm abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution (62.5 ml formalin (37%), 150 ml of sodium saline solution, 150 ml of buffer solution and 500 ml of double-distilled water) (Schaefer and Holzmann, 2000). One drop of this mixture was put on a slide and overlaid with a cover slip. The percentage of total abnormalities was determined by counting a total of 200 spermatozoa under phase-contrast microscopy (1000× magnification, oil immersion). Seminal plasma was obtained by centrifugation of semen samples at 2300 x g, 15 min at 4 °C. The seminal plasma was removed then was stored at - 60 °C until further analyses of antioxidant enzymes.

Lipid peroxidation (nmol/ml)

The assay utilized developed thiobarbituric acid-reactive substances (TBARS) which were formed as a result of reaction of thiobarbituric acid with malondialdehyde (MDA) in acidic medium at 95 ºC for 30 minutes according to Placer et al, (1966). The absorbance of the pink TBARS measured at wavelength 534 nm.

Superoxide dismutase (SOD)

The SOD activity was determined according to the method proposed by Kuthan et al. (1986) using BioAssay kit that was brought from Medibena, Austria. In the assay, superoxide (O2-) is provided by xanthine oxidase (XO) catalyzed reaction. O_2 reacts with a WST-1 dye to form a colored product. SOD scavenges the O2 thus less O_2 is available for the chromogenic reaction. The color intensity (OD440nm) is used to determine the SOD activity in a sample.

Gluthatione peroxidase (GPX)

The GPX activity was determined according to Paglia and Valentine (1967) using BioAssay Systems that improved assay to measure directly NADPH consumption in the enzyme coupled reactions. The measured decrease in optical density at 340 nm is directly proportional to the enzyme activity in the sample.

Catalase (CAT)

The CAT activity was measured according to Chiu *et al.,* (2012) using BioAssay Systems that improved assay to measure directly catalase degradation of H_2O_2 using a redox dye. The change in color intensity at 570 nm or fluorescence intensity (λem/ex = 585/530nm) is directly proportional to the catalase activity in the sample.

Jugular blood samples were collected from rams into 15 ml separation tubes and allowed to clot at room temperature for 30 minutes before centrifugation (2300 x g, 15 minutes, 4ºC). Serum was harvested from blood samples and was stored at -20 °C until measuring the levels of testosterone.

Testosterone concentration

Testosterone concentration was measured by an ELISA method (Testosterone Elisa kit, DRG International, Germany) according to Tietz, (1986).The test is based on the principle of competitive binding where endogenous testosterone of the sample competes with testosterone horseradish peroxidase conjugate for binding to the coated antibody. The unbound conjugate is then washed off and after addition of the substrate solution, the intensity of colour developed is reverse proportional to the concentration of testosterone in the sample.

Statistical analysis

Data were analyzed using the SPSS (2005) computerized program 17.0 to calculate the analysis of variance (ANOVA). Duncan multiple range test was used to evaluate the significant difference between means at $P<0.05$.

Results and Discussion

Quality assessments carried out on semen samples of M treated and C rams throughout the investigation period are presented in Table (1). It was evident from the data on semen parameters in Table 1 that M group produced semen of better quality than C group.

Ejaculate volume increased in M group from (0.64 ± 0.1 ml) at the beginning of the trial to (0.96 ± 0.15 ml) on 9th week after the first implantation. On the other hand, semen volume remained nearly stable (0.52± 0.13 ml on the 1st week of implantation and 0.57± 0.17 ml on the last week of implantation). Ejaculate volume was significantly ($P < 0.05$) higher in M compared to C rams throughout the experiment. The same trend was demonstrated for motility Score**,** sperm concentration, alive sperm %, total sperm output and abnormalities % . Significant differences ($P < 0.05$) between groups were observed in all previous parameters at 1st, 3rd and 9th weeks after the first implantation to the benefit of M group.

Table 1: Semen parameters in melatonin-treated (M) and control groups (mean ± SEM)

Groups / Parameters	Week 1		Week 3		Week 9	
	C	M	C	M	C	M
Volume (ml)	0.52^{b}±0.13	0.64^{b}±0.1	0.55^{b}±0.14	0.74^{a}±0.11	0.57^{b}±0.17	0.86^{a}±0.15
Motility Score (1-5)	3.53^{b}±0.10	4.10^{a}±0.11	3.39^{b}±0.13	4.30^{a}±0.11	3.63^{b}±0.12	4.55^{a}±0.11
Sperm Concentration (109/ml)	3.31^{b}±0.57	4.25^{a}±0.54	3.53^{b}±0.47	4.30^{a}±0.57	3.44^{b}±0.71	4.51^{a}±0.47
% Live sperm	81.6^{b}±0.63	91.60^{a}±0.34	81.80^{b}±0.40	92.20^{a}±0.33	82.40^{b}±0.61	94.50^{a}±0.45
Total sperm output (109/ml)	1.72 ^{b}± 0.37	2.72 ^{a} ± 0.31	1.94^{b}± 0.27	3.18^{a} ± 0.22	1. 96^{b} ± 0.3	3.87^{a}± 0.29
% Abnormalities	15.20^{a}±0.62	8.20^{b}±0.41	17.10^{a}±0.73	6.20^{ab}±0.24	14.80^{a}±0.41	5.20^{ab}±0.32

Means followed by different superscripts a, b between groups mean significant differences (P < 0.05).

The influences of melatonin on lipid peroxidation and antioxidant activities in ram semen are summarized Table 2. Regarding the lipid peroxidation, melatonin implantation decreased seminal MDA concentrations and lower significantly ($P < 0.05$) their levels to reach their minimal levels (2.2 ± 0.8 nmol/dl) on the 9th week and consequently improving semen quality through minimizing lipid peroxidation. Concerning the antioxidant enzymes activities, melatonin caused a significant increase ($p < 0.05$) in CAT, GPX and SOD ability compared to the control group. The activities of seminal CAT, GPX and SOD reached their maximal abilities at the end of experiment to reach 1.7 ± 0.1 U/ml, 472.6 ± 30.2 mU/ml and 60.6 ± 4.9 U/ml respectively.

Table 2: Oxidative parameters and testosterone levels in melatonin-treated (M) and control groups (mean ± SEM)

Groups / Parameters	Week 1		Week 3		Week 9	
	C	M	C	M	C	M
MDA (nmol/dl)	6.1 ± 0.7^{a}	3.2± 0.6 ^{b}	5.3 ± 0.7^{a}	2.6± 0.6^{b}	5.7 ± 0.8 ^{a}	2.2 ± 0.8^{b}
CAT (U/ml)	0.6 ± 0.2^{b}	1.3 ± 0.1^{a}	0.5 ± 0.1^{b}	1.5 ± 0.2^{a}	0.7 ± 0.1^{b}	1.7 ± 0.1^{a}
GPX (mU/ml)	421.2 ± 32.2 ^{b}	439.0 ± 27.4 ^{a}	434 ± 25 ^{b}	453.6 ± 33.9 ^{a}	425.8 ± 16.1 ^{b}	472.6 ± 30.2 ^{a}
SOD (U/ml)	15.5 ± 1.7 ^{b}	52.2 ± 6.3^{a}	17.4 ± 5.6^{b}	53.0 ± 3.3^{a}	16.6 ± 4.3^{b}	60.6 ± 4.9^{a}

Means within the same row followed by different superscripts are significantly different at p ≤ 0.05.

Testosterone levels in both M and C groups demonstrate a different pattern, with significant ($P < 0.05$) differences starting from 3rd week to the 9th week reaching the highest level in the 9th week (6.99±2.41ng/ml) as shown in Fig. 1.

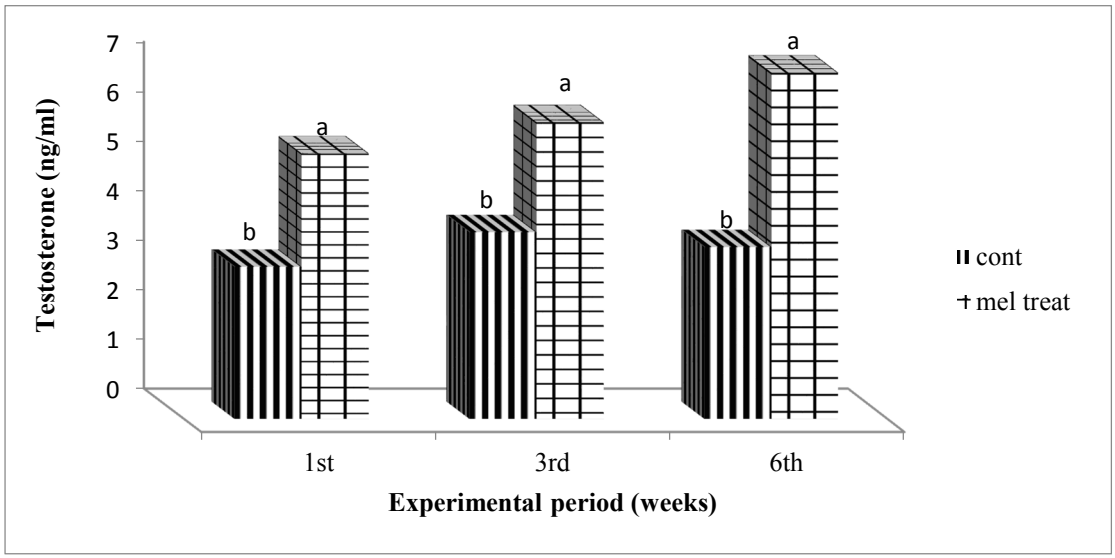

Fig. 1: Testosterone levels in melatonin-treated (M) and control groups (mean ± SEM).

Discussion

The present investigation showed the effects of melatonin implants on seminal parameters, lipid peroxidation, antioxidant enzymatic activities and testosterone levels in Tyrolean mountain rams. The current results illustrated that M group produced semen of better quality than C group as melatonin increased ejaculate volumes, improved motility Score**,** sperm concentration, alive sperm %, total sperm output and minimize abnormalities % (Table 1). The current findings are in compatible with those recorded in Manchego ram lambs by GardeLópez-Brea *et al.,* (1996) who found that melatonin implants had positive impact on seminal parameters when the implant was administered on 17th May. Moreover, our results are in agreement with Chemineau *et al.,* (1992) who found that melatonin-treated Ile-de-France and Préalpes-du-sud rams used for semen production in an AI programme had higher fertility than the control rams (67.6% versus 56.0%). The same authors reported also that Ile-de-France rams treated with melatonin produced 40% more spermatozoa and 100% more AI doses than controls. Several investigations recorded that melatonin possesed beneficial effects on preservation of mammalian sperm quality and improved the microscopic seminal parameters of spermatozoa (Rao and Gangadharan, 2008; du Plessis *et al.,* 2010; Jang *et al.,* 2010).

It was found that melatonin reduced the number of free radicals, ROS, and protected sperm cells against oxidative stressas well through stimulating the activities of antioxidant enzymes such as SOD, GPX and CAT (Karbownik and Reiter, 2000). Furthermore, Reiter (1995) concluded that melatonin was more effective than glutathione and twice as potent as vitamin E in scavenging the highly toxic hydroxyl radical and neutralising the peroxyl radical.

Concerning the lipid peroxidation, melatonin implantation decreased seminal MDA concentrations. These results are basically consistent with the results previously reported (Gavella and Lipovac, 2000).

CAT, GPX and SOD are important parts of antioxidant enzyme defense systems in sperm that convert superoxide (O2−) and peroxide (H2O2) radicals into O2 and H2O. GPX excludes peroxyl radicals from various peroxides (Irvine, 1996). CAT and SOD also eliminate O2 − produced by nicotinamide adenine dinucleotide phosphate-reduced (NADPH) oxidase (Jeulin *et al.,* 1989).

Regarding, antioxidant enzyme activity in seminal plasma, our study found significant differences in the enzymes studied. The present results are in agreement with other works (Limon-Pacheco and Gonsebatt, 2010; Rao and Gangadharan, 2008) who had shown a regulation of the glutathione system by melatonin. Marti *et al.,* (2007), observed also differences in the catalase and the SOD activity levels between reproductive and non-reproductive seasons. Moreover, Casao *et al.,* (2010b) reported a close correlation between the melatonin levels in ram seminal plasma and the catalase and SOD enzymatic activities. However, the same authors revealed no correlation between melatonin levels and GPX enzymatic activity.

As regards the testosterone, our present work showed that melatonin significantly ($P < 0.05$) increased its level after treatment. These results go parallel with those findings recorded by Casao *et al.,* (2013): Kaya *et al.,*

(2000); and Kokolis *et al.,* (2000) who found that melatonin implants induced an increase in testosterone levels in both blood and seminal plasma.

The influence of melatonin on increasing blood testosterone concentrations may be ascribed to one and/or all of the following physiological mechanisms:

I- Stimulating effect of melatonin on the hypothalamus-pituitary axis, causing the testosterone levels to rise through an increase in the GnRH and the LH secretions (Misztal *et al.,* 2002).

II- Melatonin could possibly act in the reproductive axis through direct action on the GnRH gene expression and regulation of the G-protein coupled melatonin receptors on the GnRH neurons of the hypothalamus by binding to its receptors (Roy *et al.,* 2001).

III- Direct action of the melatonin on the testosterone secretion by the testes through binding with its receptors in the Leydig cells (Frungieri *et al.,* 2005).

IV- Direct involvement of melatonin in androgen secretion (Valenti and Giusti, 2002).

Conclusion

This study reveals the action of commercial melatonin treatment of rams during the non-reproductive season on seminal profile, antioxidant enzymatic activity and testosterone levels of ram. The results indicated that melatonin improved semen parameters through its protective effects on spermatozoa and increasing activities of antioxidant enzymes. Further, melatonin also increased testosterone concentrations in the blood through various physiological mechanisms.

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