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CHANGES IN TESTES, SPERM MORPHOLOGY AND PERIPHERAL LEVELS OF FSH AND LH IN AN ADULT NEW ZEALAND MALE RABBIT EXPOSED TO DARKNESS

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Abstract

Biological rhythms control many physiological and behavioral functions in mammals, including reproduction. During the development of the reproductive system, testicular growth is primarily influenced by photoperiod, which is influenced by the circadian release of melatonin. The purpose of this study was to determine the effect of darkness on the gonadotropic axis in mature male rabbits. This study used ten male rabbits separated into two groups: a control group and a group that was placed in complete darkness for 15 days. After the sacrifice, using the IRMA method, blood samples were collected to assess serum LH and FSH hormone levels. Histopathology (testis) parameters were investigated and sperm smears were also obtained to investigate the morphological structure of the collected sperm. The results revealed morphological alterations in treated rabbits as well as a significant decrease in blood FSH and LH levels when compared to controls, suggesting a relationship between the gonadotropic axis and the pineal gland. The results of this study reveal that factors that activate the gonadotropic axis, such as darkness, can cause alterations in reproductive function, and is also dependent on photoperiod duration.

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Introduction

Several environmental factors, such as photoperiod, temperature, food, and soil type, might have an impact on the animals' well-being. Many mammalian species with photoperiodic reproductive cycles live in temperate climate zones (CHEMINEAU et al. 1992). Most mammals' reproductive functions have a seasonal rhythm, which is often under photoperiodic regulation. Animal rhythmicity, particularly circadian rhythms, is based on the interaction of physiological systems known as the internal clock or biological clock. These rhythms control an extensive variety of physiological and behavioral processes in mammals, including reproduction, so that calving occurs when environmental conditions are favorable, increasing the chance of survival for the young animals.

In comparison to hormonal treatments, light programs are less expensive and easier to implement. To reduce the negative consequences of decreasing day duration, commercial rabbit breeders in Europe adopt a 16 h light and 8 h dark (16HL: 8HD) continuous lighting cycle (ALVARINO and UBILLA 1993). The photoperiod therefore has an essential influence on animal reproduction. The cycle of reproduction of the domestic rabbit *Oryctolagus cuniculus* begins with the lengthening of the light period in spring and concludes with an increase in the number of hours of darkness during the day (LEBAS et al. 1986). During the development of the reproductive system, testicular growth is primarily determined by the photoperiod, which has a strong connection to the circadian release of melatonin (CZEISLER and KLERMAN 1999). According to LEBAS et al (1990), changing from 8 to 16 hours of light causes an increase in testicular weight and the proportion of viable spermatozoa, while changing from 8 to 16 hours of light causes a drop in these same parameters. The testis, which is composed of seminiferous tubules, is the tubular compartment responsible for the production of spermatozoa: spermatogenesis, which is primarily sustained by testosterone, which was previously synthesized by the Leydig cells (CURTIS and AMANN 1981, EURELL and FRAPPIER 2006). The rabbit is a hardy species that is regarded as an important model in scientific research because of its various advantages in the field of reproductivity, which allows particular reproductive processes to be highlighted (EWUOLA and EQUNIKE 2010).

The biological clock that determines circadian rhythmicity has been the most studied; at present, there is no complete comprehension of these distinct levels in any experimental model. A few studies have examined the influence of circadian rhythm disruption on sperm abnormalities, testicular capacity and reproductive hormone levels in rabbits to assess

the impact of darkness on the development of the reproductive system (MOUSTAFA 2020). The aim of our study is to determine the effect of photoperiod on sperm morphology and serum gonadotropin levels in mature domestic rabbits (*Oryctolagus cuniculus*) after a 15 days period of permanent darkness.

Experimental materials and methods

This study was conducted in Tizi Ouzou (a rural town) in northern Algeria. Twenty male New Zealand White rabbits, aged eight months, with a mature weight of approximately 3.5 kg, used during the study. The animals were divided into two groups: group I (control) received 8 hours of light and 16 hours of darkness (8HL : 16HD), whereas group II was subjected to constant darkness for 15 days.

To maintain the same environmental conditions, all experimental male rabbits were kept in the same rabbit house by the same breeder and large-scale rabbit farm management method. Every male rabbit was kept in a solitary cage with ad libitum access to food and water with acclimation period of 8 days. Commercial pellets (raw protein: 16.1%, crude fiber: 18%, crude ash: 12%, calcium: 1.3%; dry matter: 89.5 %; digestible protein: 13.5%; mineral matter: 7%) were supplied to the rabbits. The "Guidelines for Experimental Animals" and the institution's approved protocols were strictly followed during every step of the experimental process. Throughout the course of the experiment, no rabbit deaths or illnesses were noted. Changes in the body weight of rabbits were assessed during the experimentation.

Semens smear preparations were stained with Hematoxylin and Eosin (Sigma Aldrich). LH and FSH levels were measured by Immuno Radio Metric Assay (IRMA ; Immunotech Inc. Beckman Coulter, France).

Histological processing

Testes that were previously dissected were quickly preserved in Bouin's solution. The testes were dehydrated in a graded series of ethanol and then embedded in praffin. After slicing each block into four-micron-thick sections, hematoxylin and eosin stain (HE; Sigma Aldrich, France) was applied. An Optika B-500 TPL "TS-View" light microscope was used to examine the slides.

Smear sperme analysis

Sperm quality is usually assessed in spermatozoa collected from the cauda epididymidis of freshly sacrificed males rabbits and was performed using hematoxylin and eosin staining. Smears were prepared for morphological evaluation using slides precleaned with 70% ethanol. 5 µL aliquot of semen was placed on each slide, which was air-dried at 37°C in a warm tray. The slides were stained with Hematoxylin and Eosin.

The slides were fixed in Etathol/Methanol 95% and washed in running tap water then dried on absorbent paper. Next, the smears were stained with a hematoxylin staining solution and washed with water. The smears were stained with Eosin staining and then fixed with 96% ethanol. Slides were placed vertically to drain the excess solution and to allow them to air-dry.

All the slides were viewed with an X100 oil-immersion objective under the Optica microscope, using immersion objective lenses. The images of sperm were examined on the computer screen.

Hormone assays

The blood samples were collected into a set of sterile plastic bottles and allowed to coagulate to produce sera for hormonal analyses. Plasma follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels were measured in duplicate samples by specific IRMA methods (Immunotech Inc. Beckman Coulter, France. The volume of plasma used was 75 µl (FSH assay) and 100 µl (LH assay). All samples were measured in the same assay run to avoid inter-assay variations.

Statistical analysis

Data are presented as means ± standard deviation. Statistical comparisons between group means were performed using an Origin LAB, 2007 using Student's t-test. The level of significance was < 0.05.

Results

Compared to group I (control), group II (exposed to darkness) had a higher body weight change over the period of the experiment (15 days). In group I (control) it was evaluated at 3.70 ±0.28 kg and in group II it was 3.75 ±0.34 kg at the beginning of the experiment. At the end of the study, it was estimated at 3.89 ±0.21 kg in group I (control) and 4.35 ±0.28 kg in group II exposed to darkness for 15 days.

The microscopic analysis of cross-sections of control rats' testes reveals a normal structural appearance of seminiferous tubules surrounded by a peritubular sheath, with seminiferous epithelium containing germ cells at various stages of the spermatogenetic cycle, with cell types represented by spermatogonia, Sertoli cell, spermatocytes, round spermatids surrounded by a basal lamina and separated by interstitial tissue. The animals in the group exposed to permanent darkness for 15 days, show a significant decrease in the number of spermatozoa in the lumen of the seminiferous tubules with a large lumen, as well as fewer stages with vacuoles (Arrow) appearing among the spermatogenic cells. The tissue within the border thickened. In many tubules, the number of primary spermatocytes is either significantly reduced or eliminated. Normal spermatogenic pattern is present in very few tubules (Figure 1 – *a* and *b*).

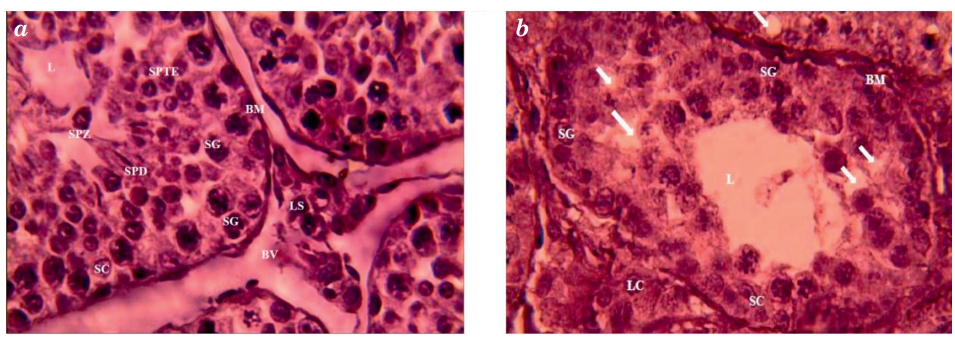

Fig. 1. Representative haematoxylin-eosin (HE) microphotographs of testicular tissue sections in rabbit (400× magnification) of control (*a*) and (*b*) under darkness. Vacuoles (arrow) appear among the spermatogenic cells

Explanations: SC – Sertoli cells; SPZ – Spermatozoa; L – Lumen; SG – Spermatogonia; SPD – Spermatide; SPTE – Spermatocyte; LC – Leidig cells; BV– Blood vessel; BM – Basement membranes

The staining of a seminal smear (Hematoxylin and Eosin) allows the qualitative evaluation of normal and abnormal sperm morphological forms in smear sperme. Smears can be scored for morphology using the World Health Organization (WHO) classification, or by Kruger's strict criteria classification (WHO 1992, KRUGER et al. 1995). WHO method, classifies

abnormally shaped sperm into specific categories based on specific head, tail, and midpiece abnormalities.

In contrast, Kruger's strict criteria classify sperme as normal only if the sperm shape falls within strictly defined parameters of shape and all borderline forms are considered abnormal (>14% normal forms).

The smear sperm analysis shows several abnormalities in the head, midpiece and flagellum, compared to the controls where most spermatozoa were normal (Figure 2).

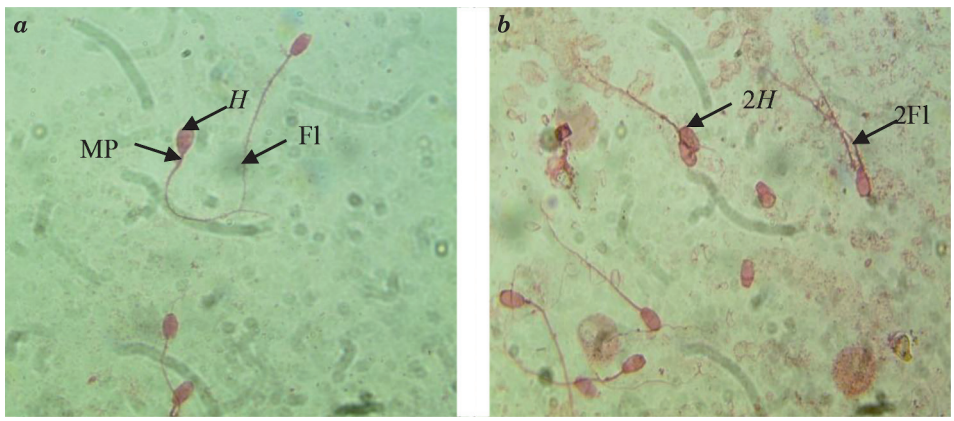

Fig. 2. Spermatozoa stained with Hematoxylin-eosin rabbit (1000× magnification) of the described Groupe I control (*a*) and Group II (*b*) under darkness
Explanations: *H* – Head; MP – Midpiece; Fl – Flagellum

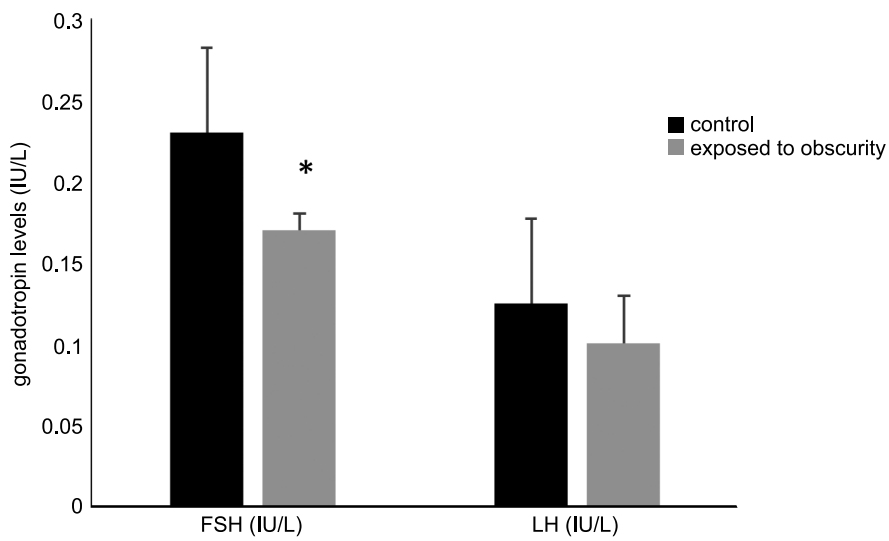

Fig. 3. Changes in the levels of gonadotropin hormone (FSH and LH) in male rabbit after prolonged dark exposure for 15 days. Values are expressed as means ±SEM
* $P < 0.05$ vs. the control group by Student's t-test

The effect of the obscurity for 15 days on the serum hormone profile of male rabbits is presented in Figure 3. The LH levels were found ranged within 0.09 to 0.120 IU/L. The obscurity had a negative impact on LH levels ($p < 0.05$) (0.10 ±0.009 IU/L V*s* 0.125 ±0.003 IU/L). During the period of obscurity, FSH levels in the Rabbit male ranged from 0.10 to 0.25 IU/L (0.17 ±0.03 IU/L V*s* 0.23 ±0.010 IU/L). The progressive decrease in FSH and LH values suggests that exposing rabbits to darkness for 15 days reduces serum circulating FSH and LH levels.

Discussion

One important hormone that influences circadian cycles and controls animal reproduction is melatonin. By stimulating receptor sites within the hypothalamic-pituitary-gonad (HPG) axis, darkness facilitates its synthesis (ALLAIN et al. 1994, SHI et al. 2013). Melatonin (also known as "the hormone of darkness") is produced throughout the night and has an impact on the synchronization of the circadian cycles of physiological functions such as growing. The effect of melatonin treatment is similar to what happens when animals spend extended periods of time in the dark. It's production by the pineal gland follows a circadian cycle, with low levels during the day and high levels at night (BRZEZINSKI 1997). Testis and epididymis weights, as well as daily sperm production and ovary weights, revealed significant seasonal change in the Mediterranean climate, with peak values in March and April (GONÇALVES et al. 2002). Its plays an essential function in reproduction in addition to its role in protecting against cellular damage (REITER et al. 2000) by enhancing the activity of antioxidant enzymes and removing free radicals, particularly in the female and male gonads using receptor sites within the hypothalamic-pituitary-gonad (HPG) axis (SHI et al. 2013 ; LAMPIAO and PLESSIS 2013). Melatonin has been shown in animal studies to impact testicular function and there can be evidence that the pattern of melatonin secretion, which is controlled by photoperiod, has a direct impact on reproductive function. Seasonally reproducing mammals have provided much of the evidence (MALPAUX et al. 1999, YU et al. 2018).

On the other hand, melatonin, has been shown to inhibit reproductive activity particularly, Leydig cells in mice and rats (NG and LO 1988, PERSENGIEV et al. 1991, RASHED et al. 2010). There are contradictory research findings regarding melatonin's effect on spermatozoa function. In seasonally breeding Syrian hamsters, day length altered copulatory behavior, as males stopped ejaculating after many weeks of being exposed to short day lengths (POWERS et al. 1989, MIERNICKI et al. 1990).

The seminiferous tubule wall was thicker. These findings might indicate the increase in number of spermatogenic cell lineage, which could be the consequence of endogenous melatonin's outcome after exposure to permanent darkness for 15 days on Sertoli cells of the spermatic tubules affecting them directly through melatonin receptors found in almost all tissues and cells (RASHED-MOURAD and al. 2010, MOHAMMED and al. 2016). Several dividing spermatogenic cells degenerate as a result of the vacuoles, which arise among the spermatogenic cells, reveal different levels of damage, and disrupt distribution throughout the germ cells (RASHED-MOURAD and al. 2010). Additionally, FRUNGIERI et al. (2005) found that melatonin acts on the interstitial cells of the testes, increases the expression of testicular melatonin receptors, decreases the expression of important enzymes involved in the synthesis of steroids, inhibits the secretion of androgens, and lowers reproductive performance.

In our study, exposure to permanent darkness for 15 days appeared to affect most reproductive characteristics in male rabbits. Sperm morphological modifications are defined by morphological anomalies in the spermatozoa's several sections, including the head, midpiece, and flagellum. Our results were confirmed by LUBOSHITZKY et al. (2002) who showed that melatonin's effect on spermatozoa function has been reported to be variable. The administration of Melatonin to healthy males over time has been associated with lower sperm quality and a significant impact on sperm concentration, motility and testosterone levels in healthy men. An in vitro study, on the other hand, discovered the administration of melatonin to human spermatozoa, increased progressive motility and decreased the number of static cells (ORTIZ et al. 2011). This suggests that exposure to excessive levels of melatonin (permanent darkness) may be the cause of reproductive system damage. Mild azoospermy and oligozoospermy have been linked to high levels of endogenous melatonin in semen. Low levels of melatonin in the semen, however, are linked to aberrant sperm development (YIE and al. 1991) probably through the inhibition of aromatase at the testicular level (LUBOSHITZKY et al. 2002).

Serum FSH and LH levels in exposed rabbits to permanent darkness were significantly lower than in controls. Melatonin treatment inhibited the production of FSH and LH in male rats, affecting sexual maturation via decreasing FSH activation of Sertoli cells (LI and ZHOU 2015). Melatonin has been shown in vitro to suppress the stimulation of LH release by luteinising hormone-releasing hormone (LHRH) in pituitary cells from rat foetuses (HATTORI et al. 1995, LI and ZHOU 2015). Changes in Ca^{2+} concentrations and cAMP accumulation may be associated with melatonin-induced declines in LH production by pituitary cells (VANECEK 1998).

Ca^{2+} inflow or concentrations in pituitary cells increased the release of GnRH-induced LH, while melatonin administration partially inhibited this effect. Melatonin treatment partially inhibited this response, implying that melatonin's suppression of LH release is mediated by melatonin. Melatonin's effects may be mediated by decreases in intracellular concentrations of these second messengers. This might explain the decline in LH and FSH levels in rabbits exposed to complete darkness for 15 days.

Finally, natural light and artificial illumination have a variety of effects on reproductive parameters in farmed rabbits. Permanent darkness for 15 days results in high melatonin levels, which affects reproductive function (sperm quality). This exploratory experiment, however, does not explain the reproduction characteristics that are directly influenced by permanent darkness.

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