

# The effects of photoperiod on testes in rat: A morphometric and immunohistochemical study

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## Abstract

**OBJECTIVE:** The aim of this study was to examine the effects of photoperiod on testes in rat.

**DESIGN:** For this purpose 21 male Wistar rats were used. Animals were divided into three groups. Control rats in group I were kept under 12 hrs light: 12 hrs dark conditions (12L: 12D) for 10 weeks. Animals in group II were exposed to long photoperiods (18L: 6D), while rats in group III were exposed to short photoperiods (6L: 18D) for 10 weeks. At the end of the experiment, all animals were killed by decapitation and blood samples were obtained. Serum testosterone levels were determined with the use of a chemiluminescent enzyme immunoassay. The testes of all rats were removed and weighed. Testicular tissue was processed semiquantitative evaluation of immunohistochemical testosterone staining. Intensity of immunostaining was determined on a scale between 0 (no staining) and 5 (heavy staining). For morphometric comparison, diameters of seminiferous tubules in each group were measured.

**RESULTS:** In rats exposed to long photoperiods, testicular weights, diameters of seminiferous tubules and serum testosterone levels were significantly increased as compared to those in control rats. Whereas, exposure of rats to short photoperiods resulted in significantly decrease of testicular weights, diameters of seminiferous tubules and serum testosterone levels as compared to those in control rats and rats maintained in long photoperiods. Immunostaining of testosterone was moderate (3+) in control rats, heavy (5+) in rats exposed to long photoperiods and minimal (1+) in rats exposed to short photoperiods.

**CONCLUSIONS:** The results of our study indicate that testicular functions increase after exposure to long photoperiods and decrease after exposure to short photoperiods.

## Introduction

Diurnal rhythmicity is an important feature of the mammalian endocrine system, clearly visible in secretion of hormonal products of the pituitary and adrenal glands, but most prominent in the function of the pineal gland. In the majority of investigated mammals melatonin secretion by the pineal gland is low during day and increases markedly at night [1].

It is generally believed that in mammals the rhythm of melatonin synthesis and secretion by the pineal gland is generated endogenously by the circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. This endogenous rhythm is entrained by environmental light acting via the retina and the neuronal pathways connecting the retina with the hypothalamus. The inhibitory effect of the light on the activity of the SCN together with the intrinsic rhythmicity of the SCN ensure that melatonin is produced and secreted only during nocturnal darkness [1, 10].

In many mammals, the pineal gland is involved in the regulation of the hypothalamo-hypophysial-gonadal axis by circadian patterns of melatonin secretion [1]. The data that are available in the literature suggest that melatonin has a negative effect on testicular functions [4]. These inhibitory effects of melatonin are primarily exerted via the hypothalamo-hypophysial axis [16, 28, 34]. Melatonin receptor sites have been visualized in the hypothalamus [36]. It has been reported that melatonin down regulates gonadotropin-releasing hormone (GnRH) induced luteinizing hormone (LH) release from the anterior pituitary gland [35]. However, it has recently been suggested that melatonin can directly act at the gonadal level [31]. Melatonin may affect androgen secretion by direct action on Leydig cells [33].

Therefore, photoperiod (day length) is a potent environmental stimulus that affects reproduction. Long photoperiods stimulate reproductive development in juveniles and maintain gonadal function in adults, whereas short photoperiods inhibit reproductive development and induce gonadal regression [6-8]. It has been previously reported that long photoperiod or short photoperiod changes both the function and morphological structure of the testes [2, 3, 9, 11-13, 17-21, 22, 24, 27, 29, 30, 32, 37, 38].

In the present study, we have examined morphometrically and immunohistochemically the effects of long photoperiods and short photoperiods on testes in rat.

## Material and Methods

Adult male Wistar rats (weighing 180-200 g, n = 21) were used in this study. The animals were divided into three groups. Control rats in Group I (n=7) were kept under 12 hrs light: 12 hrs dark conditions for 10 weeks. Animals in Group II (n=7) were exposed to long photoperiods (18 hrs light: 6 hrs dark conditions, 18L:6D), while rats in Group III (n=7) were exposed to short photoperiods (6 hrs light: 18 hrs dark conditions, 6L:18D) for 10 weeks. During the whole experiment the

animals were kept at a constant temp ( $21 \pm 1^\circ\text{C}$ ). Food (standard pellet diet) and tap water were supplied *ad libitum*.

### Serum testosterone assay

Animals were anesthetized with ether and killed by decapitation at the end of the experiments. Blood was collected and serum was separated and stored at  $-20^\circ\text{C}$  for analysis. Total serum testosterone levels were measured with the use of a chemiluminescent enzyme immunoassay (Immulite Testosterone; Immulite, Los Angeles CA, USA).

### Light microscopic procedure

The testes of all rats were removed immediately after collection of the blood. The testes were dissected from the surrounding tissue and weighed. Then, testicular tissue specimens were fixed in Bouin's solution and embedded in paraffin. Paraffin sections (thickness,  $5\mu\text{m}$ ) were stained with hematoxyline-eosine. For morphometric comparison, in these sections stained with hematoxyline-eosine, diameters of 100 seminiferous tubules from each group were measured with ocular micrometer adapted to a microscope and the mean diameter of seminiferous tubules of each group was determined.

### Immunohistochemical procedure

For immunohistochemical testosterone staining of the cytoplasm of Leydig cells, paraffin sections (thickness,  $5\mu\text{m}$ ) were deparaffinized in xylene, hydrated and then placed in phosphate buffered saline (PBS; pH 7.6). Antigen retrieval was performed by boiling for 15 min in citrate buffer (0.01 M). Sections were treated with 3% hydrogen peroxide for 5 min to quench endogenous peroxidase activity, rinsed with deionized water and then washed with PBS. Sections were incubated first with 1% pre-immune rabbit serum to reduce non-specific staining and then with a monoclonal antibody against testosterone (Bio-Genex, San Ramon CA, USA) at  $23^\circ\text{C}$  in a moist chamber for 1 h. Detection of the antibody was performed using a biotin-streptavidin detection system (Bio-Genex, San Ramon CA, USA) with 3-amino 9-ethyl carbazole (AEC) as chromogen (Dako, Carpinteria CA, USA). Sections were counterstained with Mayer's hematoxylin, dehydrated and then cover-slipped with permount.

Immunohistochemical testosterone staining of the cytoplasm of Leydig cells was evaluated semi-quantitatively. Intensity of immunostaining was scored as follows: no staining (0), minimal (1+), low (2+), moderate (3+), strong (4+), heavy (5+).

### Statistical analysis

Data (testicular weights, diameters of seminiferous tubules and serum testosterone concentrations) were analyzed with the use of SPSS software for Windows. Repeated measure analysis of variance (one-way ANOVA) tests were used to test significance of differences between groups. Levels of significance were taken at  $p < 0.05$ .

## Results

In rats exposed to long photoperiods, testicular weights and diameters of seminiferous tubules were significantly increased as compared to those in control rats (Table 1, 2). Furthermore, an increase in serum testosterone levels was seen in this group (Table 3).

Whereas, exposure of rats to short photoperiods resulted in significantly decrease of testicular weights and diameters of seminiferous tubules as compared to those in control rats and rats maintained in long photoperiods (Table 1, 2). Additionally, serum testosterone levels were decreased as compared to those in group I and group II (Table 3).

In the process of immunohistochemical staining that was performed in order to have the testosterone hormone visible in testicular tissue and to investigate it, an evaluation was made according to the density of observed staining. The more testosterone antigen (Testosterone hormone) present in the tissue or in the cell, the more binding will occur and as a result, the darker staining will be established.

Immunohistochemical staining of testosterone was performed to correlate the content of testosterone in Leydig cells with serum levels. Immunostaining of testosterone was moderate (3+) in control rats (Fig. 1), heavy (5+) in rats exposed to long photoperiods (Fig. 2) and minimal (1+) in rats exposed to short photoperiods (Fig. 3).

In sum, testicular functions and synthesis of testosterone increased after exposure to long photoperiods and decreased after exposure to short photoperiods.

## Discussion

Photoperiod is a potent environmental stimulus on reproductive functions. It has been suggested that long photoperiods stimulate reproductive development in juveniles and maintain gonadal function in adults, whereas short photoperiods inhibit reproductive development and induce gonadal regression [6-8]. Decreased reproductive responsiveness to the effects of short photoperiods on gonadal regression is due to changes in melatonin production. This is because melatonin is released during the dark phase of the day by the pineal gland and it has inhibitory effects on gonadal functions [1, 4].

Results from previous experimental studies investigating the effects of photoperiod on testes indicated that, long photoperiod causes an increase in testicular weights and serum testosterone levels [21, 24]. In the present study, in agreement with the previous studies [21, 24], it was also observed that exposure to the long photoperiods caused increase in testicular weights as well as increasing serum testosterone levels.

In a study by Porkka-Heiskanen et al. [25] it was determined that, there was an increase in hypophysial LH content and serum testosterone levels after exposure to the long photoperiods in rats. Johnson et al. [21] reported that there was an increase in diameters of seminiferous tubules and number of Leydig cells of

**Table 1.** Mean  $\pm$  SEM weights of the testes (gr/100 gr body weight) in control rats and animals exposed to long photoperiod and short photoperiod. \*, significant difference as compared to control rats ( $p < 0.001$ ) using one-Way ANOVA.

Groups	Mean $\pm$ SEM
Control (12L:12D)	1.101 $\pm$ 0.012
Long photoperiod (18L:6D)	1.204 $\pm$ 0.048 *
Short photoperiod (6L:18D)	0.994 $\pm$ 0.005 *

**Table 2.** Mean  $\pm$  SEM diameters of seminiferous tubules (micrometer) in control rats and animals exposed to long photoperiod and short photoperiod. \*, significant difference as compared to control rats ( $p < 0.001$ ) using one-Way ANOVA.

Groups	Mean $\pm$ SEM
Control (12L:12D)	286.41 $\pm$ 12.35
Long photoperiod (18L:6D)	318.35 $\pm$ 16.44 *
Short photoperiod (6L:18D)	265.53 $\pm$ 15.04 *

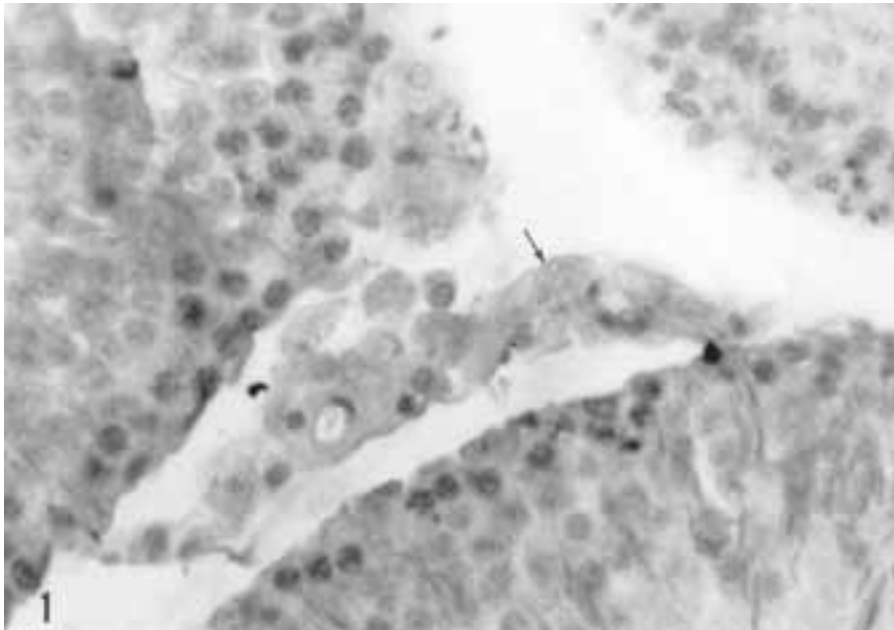
**Table 3.** Mean  $\pm$  SEM serum testosterone levels (ng/dl) in control rats and animals exposed to long photoperiod and short photoperiod. \*, significant difference as compared to control rats ( $p < 0.001$ ) using one-Way ANOVA.

Groups	Mean $\pm$ SEM
Control (12L:12D)	342.7 $\pm$ 21.50
Long photoperiod (18L:6D)	438.2 $\pm$ 35.08 *
Short photoperiod (6L:18D)	211.8 $\pm$ 24.05 *

hamsters, after 12-weeks exposure to the long photoperiods. Additionally, their electron microscopic investigations yielded that there were enlargement in smooth endoplasmic reticulum, golgi complex and increase in number of mitochondria in the Leydig cell cytoplasm, indicating increased cellular activity. Increase in the diameters of the seminiferous tubules was also observed in the present study after exposure to the long photoperiods. By immunohistochemical staining we also determined increase in testosterone synthesis in Leydig cells after exposure to this long photoperiods. Our study is in agreement with the previous studies with this respect [21, 25].

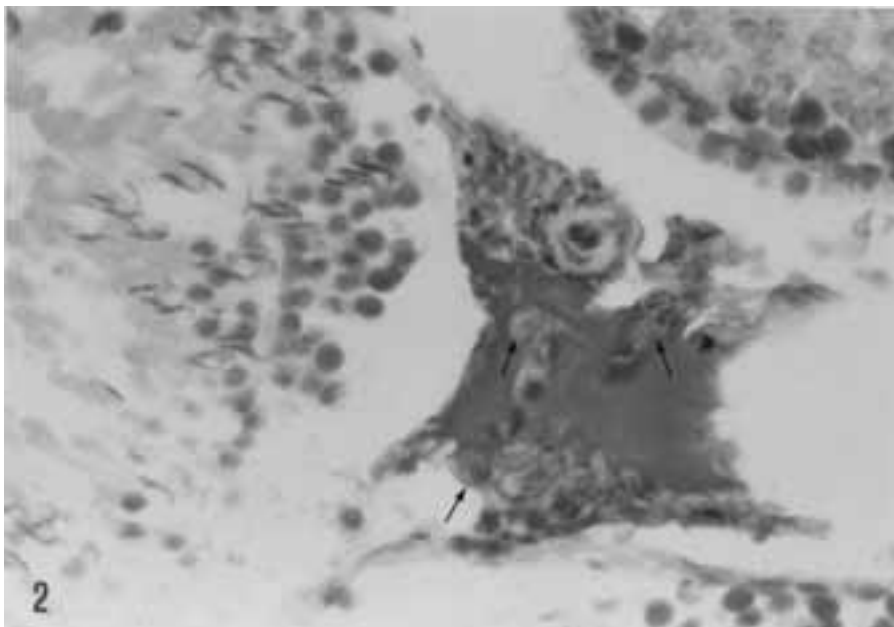
Exposure to constant light or long photoperiods suppresses melatonin synthesis in the pineal gland and this removes (diminishes?) the inhibitory effect of melatonin on gonads, which leads to increase in gonadal functions [1]. Previous studies reported inhibitory effects of melatonin on the hypothalamo-hypophysial gonadal axis and increased gonadal functions after removal of the pineal gland [5, 10, 26].

In the study of Hagen and Asher [15], an increase in activity of Leydig cells and spermatogenesis was found after pinealectomy in hamsters. In a previous study, we reported an increase in testicular weight and hyperactivity of the Leydig cells after pinealectomy on the basis of an extended Golgi complex, enlarged smooth



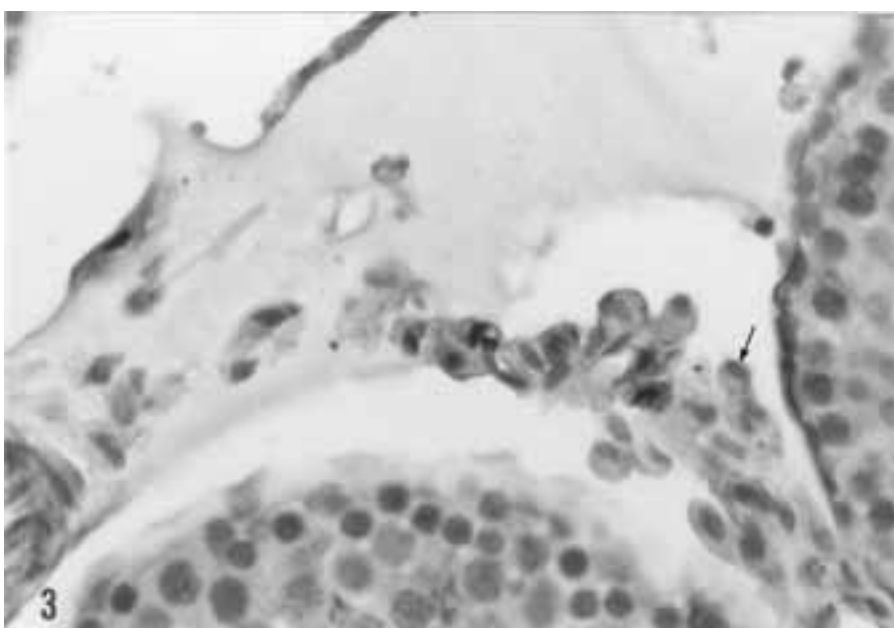
**Fig. 1.** Immunohistochemical staining of testosterone in control testis, showing moderate levels of testosterone in the cytoplasm of Leydig cells (arrow). Magnification, X40.

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**Fig. 2.** Immunohistochemical staining of testosterone in testis of rat exposed to long photoperiods, showing strong testosterone staining in the cytoplasm of Leydig cells (arrows). Magnification, X40.

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**Fig. 3.** Immunohistochemical staining of testosterone in testis of rat exposed to short photoperiods, showing low levels of testosterone in the cytoplasm of Leydig cells (arrow). Magnification, X40.

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endoplasmic reticulum and higher amount of mitochondria in Leydig cells in testes of pinealectomized rats. Increased formation of cytoplasmic secretory granules was also found [23]. Thus, findings from the study of Hagen and Asher [15] and our previous electron microscopic study [23] are supporting the findings of the present study in which we have investigated the effects of long photoperiods exposure on testes.

On the other hand, exposure to constant darkness or short photoperiods led to atrophy in gonads by stimulating melatonin synthesis in the pineal gland [10]. Previous studies have shown decrease in testicular weights and serum testosterone levels following exposure to short photoperiods [2, 3, 9, 11-13, 17, 20-22, 24, 29, 32, 37]. In the present study it was determined that the testicular weights and serum testosterone levels of the rats exposed to the short photoperiods was significantly lower than those of controls.

Exposure to constant darkness has been shown to cause decrease in diameters of seminiferous tubules associated with inhibition of spermatogenesis in rats [13]. Scholatt et al. [30] reported that restriction of light inhibited spermatogenesis in hamsters. In another study by Hikim et al. [18] it was determined that following 12-weeks exposure to short photoperiods there was a decrease in diameters of seminiferous tubules and Leydig cell counts in hamsters. Additionally, after housing the hamsters in darkness in 23 hours of the day for 9 weeks Reiter et al. [27] reported involution in testes and accessory sex organs associated with decrease in testicular weights. Hoffman and Reiter [19] also reported atrophy in gonads after exposure to 23 hours daily darkness period for a month in male hamsters. It was also determined that, there was involution in seminiferous tubules and decrease in the diameters of the seminiferous tubules in testes after bilateral optic enucleation in hamsters [14]. In an electron microscopic study by Hikim et al. [17] it was also reported that the size of Leydig cells nuclei, number of mitochondria and secretory granules were decreased following exposure to short photoperiods in hamsters, indicating decreased Leydig cell activation. Additionally, housing of hamster in 19 hours daily darkness periods for 12 weeks, was reported to cause decrease in diameters of seminiferous tubules and number of Leydig cells [21]. It was also reported that exposure of the mice to the 16-hourly daily darkness for 8 weeks caused regression of testes, decrease in serum testosterone levels and increase in apoptotic cell death [38]. In the present study we also determined decrease in the diameters of the seminiferous tubules following exposure to short photoperiods in rats. Additionally a decrease in synthesis of testosterone in Leydig cells was immunohistochemically determined in this study. Our findings are in agreement with the previous studies.

On the basis of our morphometric, immunohistochemical and biochemical findings, it is concluded that testicular functions and synthesis of testosterone increase after exposure to long photoperiods and decrease after exposure to short photoperiods.

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