Continuous darkness provokes testicular structural modification in mature rats

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Abstract: Background: The Testis is the main genital male organ that possesses both exocrine and endocrine gland roles. Melatonin is the code hormone of the pineal gland which is essentially produced during period of darkness and it is recognized to reach almost all bodily cells and organs regulating their function, especially the reproductive organs. **Aims:** This work was designed to study the effect of increasing periods of continuous darkness on testicular tissues. **Methods:** Eight groups of adult Wister albino rats were kept in complete 24 hours darkness intended for 4 successive periods. Groups II, III, IV and V were put in continuous darkness for two, four, six and eight weeks successively. Group I^a, Group I^b, Group I^c, and Group I^d were the control groups for groups II, III, IV and V consecutively. After the last day of the dark period for each group, the rats were killed under effect of anesthesia. The right testis was taken, weighed and processed for morphometric as well as anatomical and histological study. **Result:** The results showed no important structural effect on short and medium periods of darkness, while on long periods; there were significant effects which were proportionate to the time length of darkness.
Conclusion: The continuous darkness affects the testicular tissues of rats; hence, the reproduction status, depending on the length of exposure.
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1. Introduction

The pineal gland is more or less a fundamental organ in the brain. Melatonin is a natural neuro-hormone secreted by pineal body. It orders the goings-on of antioxidant, neuro-endocrine and other structures. At the day the pineal gland is at its lowest level of activity. When the darkness comes, the pineal begins to active release of melatonin hormone into the blood stream. So melatonin is sometimes named as the 'Dracula of hormones' because it is only released during the darkness. In the morning, when light is perceived, melatonin secretion is stopped, which stimulates the secretion of other hormones. Thus other systemic activities start. This regular daily rhythm is at top of importance to bodily physiology, intellectual abilities, and emotional welfare [1, 2 & 3]. The pineal gland and its main hormone namely melatonin are the body's main timekeepers giving information about the time of day, season of the year, and living phase to the brain and all the body. Regulation of this rhythm also gives the idea that the body to work for longer time, making it efficient and more balanced. Hence, is not only the essential chemicals regulator, but the body will not age faster. Melatonin is believed to affect all of the internal physiological processes and if this internal biological clock system becomes disorganized in any way, the body will be more susceptible to any harmful status [3].

In this work; incremental periods of continuous darkness were used to find out their effect on testicular biological activity in adult male rats, though a previous Iraqi study was done, using only one period of darkness instead of ongoing increasing periods of continuous darkness to find out an analogous effect [4]. The biological activity of testes in the instant study, of both exocrine activities, "*indicated by seminiferous tubules*" with endocrine activity "*revealed by Leydig cell*" in the ongoing study [5]. Nevertheless, the whole male genital system structure and activity depends upon testosterone as a core leader. Thus it is a very dependant relationship between endocrine and exocrine compartments of

testis, since testosterone is predominantly secreted by Leydig cells [5 & 6].

Aim of the study:

The aim of the research at hand is to study the *"histological modification"* in testicular tissues, in response to gradually increasing periods of constant darkness in mature male rats.

2. Materials and Methods

Adult fit male 10 weeks old Wister albino rats, forty in number were used in this study. This project was achieved within a course of the time from the morning of 9th of December of 3013, till the morning of 13th of January of 2014. They were placed in a controlled animal room, belonged to postgraduate researches, at the department of anatomy, histology and embryology, College of Medicine, Al-Mustansiriyah University, Baghdad, Iraq. The experimental rats were managed according to National Center for Control and Pharmaceutical Research, Baghdad, Iraq, which follow legislation for the protection of animals used for scientific purposes. They were divided into 8 groups, each contained 5 rats. Each rat was kept individually in a wire meshed stainless steel cage, room temperature was of 22 ± 2 ^{0}C, fed controlled pellet diet and tap water was provided for drinking *ad libitum.* Group I^a, Group I^b, Group I^c, and Group I ^{d} were put on 12:12 light – dark cycle. They were the control groups for groups II, III, IV, and V sequentially. Groups II, III, IV, and V were put in continuous darkness for a period of 2, 4, 6 and 8 weeks respectively. At the last day of 1st couple of weeks rats of group II with its control group (Group I^a) were sacrificed under effect of diethyl ether anesthesia, the whole right testis was weighed after removal of all nearby tissues and as much as possible the perinephric fat; using a dissecting microscope, then fixed in Bouin's solution immediately and processed through……? for histological study by a light microscopy, using serial paraffin sections of 5 µm thickness stained with haematoxyline and eosin [7]. In the same manner the rats of group III were dissected at the end of the 4th weeks also with its control group (Group I^b). The rats of group IV with its control group (Group I^c) were managed in the same way at end of the 6th week, and rats belong to group V with its control group (Group I ^{d}) were operated on at end of the 8th week. Histological as well as anatomical examinations were performed. Histological study was done both as descriptive and morphometric. The stereological analysis was estimated by using Zeiss Integrating Micrometer – disk Turret I of 25 point system, used on a light microscope, the total points falling on each of the testicular tissue components i.e. seminiferous tubule wall, lumen and interstitial spaces were estimated. From each section 5 fields were taken randomly, examined at 150X magnification [8]. Data were taken as mean ± SD of 5 rats. Biostatical analysis was done to evaluate the significance of results by analysis of variance, using student-t-test [9].

3. Results

Body weight was affected in all groups, taking in regard the natural gain in weight in growing animal with time according to age (Table 1). Anatomically the testis weight was affected in a variable degree in all groups. Testis weight was increased incrementally with the increase in period of darkness, then after, the weight decreased in the last group (Table 2). Table 3 shows the difference in testicular weight in 100 gm body weight in autopsy.

Histological assessment noted the following morphometric results:

(1) The number of points overlying the wall of seminiferous tubules (which refers to the wall thickness), was significantly increased progressively, with the increase in length of dark period till group V; at what time the number decreased.

(2) The number of points falling on the lumens of the seminiferous tubules followed the same manner as that of the wall, i.e. it increased incrementally with all tested groups except the last group (group V) where an apparent reduction in number of points, was noticed.

(3) The number of points superimposed on the interstitial spaces in the same examined surface area followed a reverse manner to that of the serminiferous tubules parameters.

The three parameters were expressed in Table4.

As regarding the descriptive histological result, the following findings were discerned:

Cells of spermatogenic lineage were examined in each group. They were more abundant in groups II, III and IV especially; spermatogonia (both of dark and pale type), also primary spermatocytes, spermatid and spermatozoa (both developing and mature ones) were noticed abundant. Also groups II, III and IV especially showed an increasing frequency of apoptotic and pyknotic cells. Mitotic figures in spermotogenic cells were demonstrated evidently in groups II, III and rarely in group IV [Figs.1, 2 & 3].

In the group V there was increase in the interstitial stromal Leydig cells though less frequent than in other previous groups but they appeared to be larger. Some patchy areas of interstitial spaces were almost completely changed to fibrotic tissues, with a few cells of Leydig with fibroblasts and epithelioid cell infiltration [Fig.4].

Few seminiferous tubules were seen with thickened basement membrane, consisting of hyalinized connective tissue and those seminiferous tubules showed Sertoli cells mainly, however, still retaining a few cells of spermatogenic lineage. Also in

this group multinucleate spermatogenesis cells were seen. Though some recognized seminiferous tubules lacked the spermatogenic cells, the lumen of which was obliterated completely by Sertoli cells [Fig.5].

In all groups except group V; Sertoli cells were not clearly identified, hence it was so difficult to be studied.

Blood vessels dilatation was obvious in all treated groups; and the degree of dilatation proportionate positively with the amount of melatonin used.

Leydig cells were identified by their polygonal or round shape. The nucleus was central, vesicular and round with prominent nuclear membrane having one or two nucleoli. The cytoplasm was almost granular, pale eosinophilic with some intra-cytoplasmic, eosinophilic, elongated or rectangular otherwise rhomboid masses.

Some of Leydig cells showed yellow-brown pigmented dots in their cytoplasm, while the epithelioid cells were identified by their voluminous pinkish cytoplasm, with pale eccentric nucleus. The fibroblasts were characterized by rich irregularly branched basophilic cytoplasm, with oval central pale nucleus and distinctive nucleoli.

Table 1: The effect of continuous darkness on body weight (in grams) of 10 wk old male rats.

Time of keeping rats in continuous darkness	Body wt of rats in grams at 1st day of experiment	Body wt of rats in grams at last day of experiment	Difference in body wt in grams
Control (Group I[a])	411.17±62.9	422.95±74.9	Added 11.78±0.9
2 wk **continuous darkness**	414.84±77.1	428.54±75.2NS	Added 13.17±1.6NS
Control (Group I[b])	410.97±65.1	431.42±51.1	Added 20.45±0.8
4 wk **continuous darkness**	422.03±82.9	432.11±80.2*	Added 10.08±0.9*
Control (Group I[c])	423.23±44.0	454.56±79.0	Added 31.33±1.2
6 wk **continuous darkness**	416.00±72.8	408.09±90.1*	lost 7.91±0.9*
Control (Group I[d])	418.02±74.6	457.19±75.2	Added 39.17±2.0
8 wk **continuous darkness**	419.25±64.1	402.25±87.7**	Lost 17.00±1.4**

-Results were expressed in mean ± SD of 5 rats.
--The difference of each group was statistically significant (*P<0.03, **P<0.01)) when compared with its control, NS=not significant (P>0.05).

Table 2: The effect of continuous darkness on right testis weight (in mg) of 10 wk old rats.

Time of keeping rats in continuous darkness	Right testis weight (mg) at autopsy
Control (Group I[a])	1519.22±62.3
2 wk **continuous darkness**	1521.24±51.3NS
Control (Group I[b])	1524.19±73.3
4 wk **continuous darkness**	1556.41±49.2*
Control (Group I[c])	1527.51±61.7
6 wk **continuous darkness**	1569.52±56.3*
Control (Group I[d])	1549.13±71.6
8 wk **continuous darkness**	1451.17±51.2**

-Results were expressed in mean ±SD of 5 rats.
-The difference of each group was statistically significant (*P<0.02, **P<0.001)) when compared with its control, NS=not significant (P>0.05).

Table 3: The effect of continuous darkness on right testis weight to body weight ratio in 10 wk old male rats.

Time of keeping rats in continuous darkness	Testis weight per 100g body weight
Control (Group I[a])	359.19±26.2
2 wk continuous darkness	354.98±31.1 NS
Control (Group I[b])	353.29±25.6
4 wk continuous darkness	360.18±26.5 NS
Control (Group I[c])	336.04±27.2
6 wk continuous darkness	384.60±22.2**
Control (Group I[d])	338.80±21.9
8 wk continuous darkness	361.66±18.7*

Results were expressed in mean ± SD of 5 rats.
-The difference of each dose group when compared with its control, was statistically significant (*P<0.03), highly significant (**P<0.004), or NS=not significant (P>0.05).

Table 4: Number of points overlying the seminiferous tubule wall and lumen, as well as the interstitial space, in testis of adult rat in response to continuous darkness (in unit area of 0.0025mm²).

Time of keeping rats in continuous darkness	Points on seminiferous tubule wall	Points on seminiferous tubule lumen	Points on Interstitial space
Control (Group I[a])	15.21±1.3	4.26±1.9	7.53±0.17
2 wk continuous darkness	15.92±0.9 NS	5.89±1.8 NS	6.88±0.23 NS
Control (Group I[b])	15.33±1.3	4.24±1.7	7.53±0.18
4 wk continuous darkness	16.31±1.5*	6.13±0.9*	6.15±0.19 NS
Control (Group I[c])	15.18±1.9	4.32±1.2	7.53±0.16
6 wk continuous darkness	16.71±1.83**	6.91±1.31*	5.97±0.12**
Control (Group I[d])	15.24±1.7	4.56±1.9	7.53±0.14
8 wk continuous darkness	13.12±0.8**	2.08±1.7***	8.34±0.11**

-Data were expressed as mean ± SD of 5 rats.

-When any group was compared with its control, the difference was statistically either significant: (* P<0.05, ** P<0.02, ***P < 0.008); or NS=not significant (P>0.05).

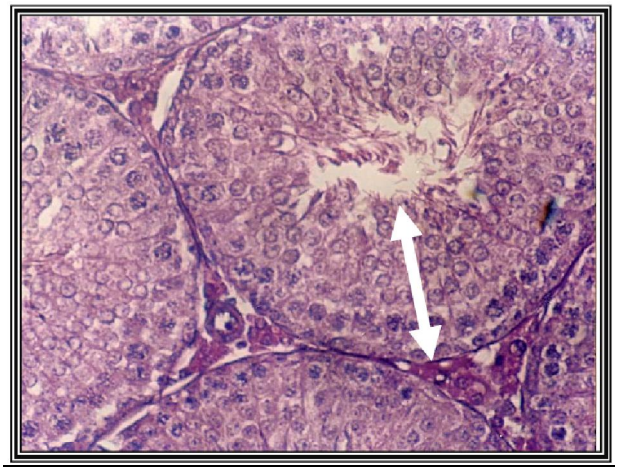

Figure 1: Photomicrograph of testicular tissues in control rat, the average wall thickness was measured (double head white arrow); H&E stain X250

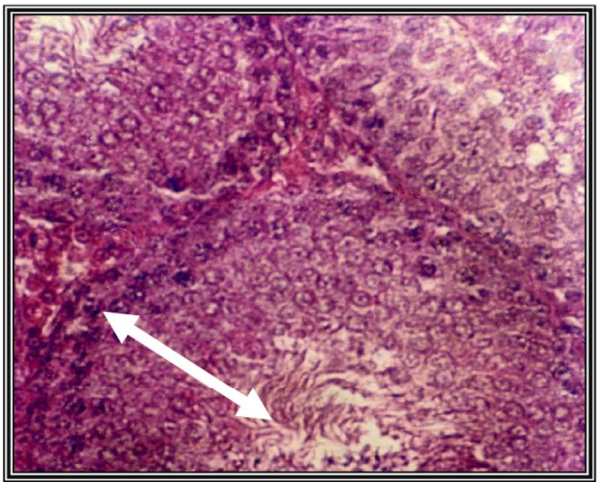

Figure 2: Photomicrograph of testicular tissues in rat kept in continuous darkness for 4 wk, the average wall thickness was increased (double head white arrow); (H&E stain X250).

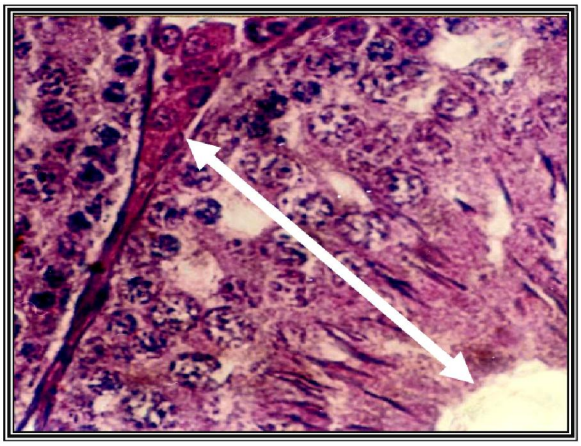

Figure 3: Photomicrograph of testicular tissues in rat kept in continuous darkness for 4 wk, the wall thickness was increased (double head white arrow) due to increase in spermatogenic lineage cells, in the seminiferous tubule (H&E stain,×400).

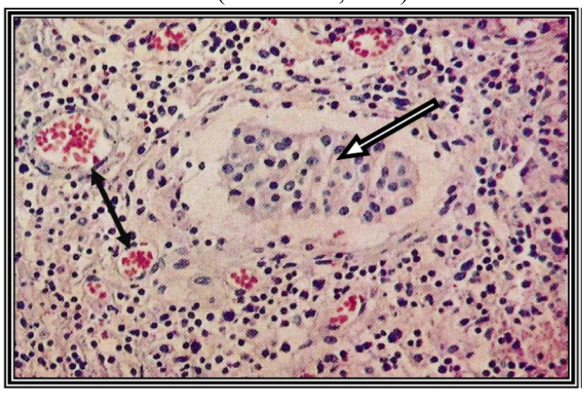

Figure 4: Photomicrograph of testicular tissues in rat kept in continuous darkness for 8 wk: some regions showed severe damaged manifestation and large fibrotic areas were clear with heavy infiltration of inflammatory cells. The seminiferous tubule seen here contains Sertoli cells mainly (arrow). Dilated blood vessels were seen (double head arrow); (H&E stain,×100).

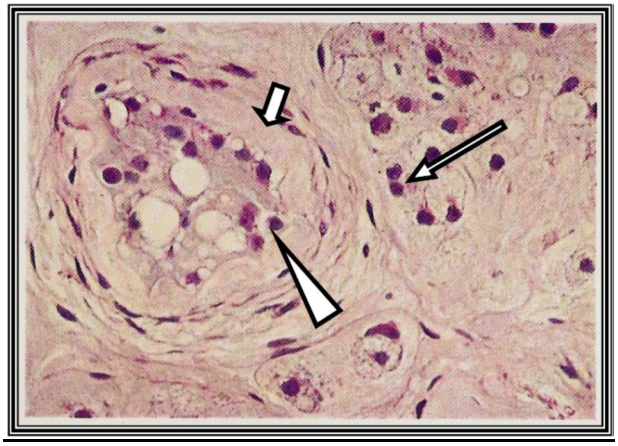

Figure 5: Few of seminiferous tubules were viewed with thickened basement membrane, consisted of connective tissue and those seminiferous tubules showed Sertoli cells mainly(short arrow), however, still retaining a few cells of spermatogenic lineage (arrow head). Also in this group multinucleate spermatogenesis cells were seen (long arrow). Though some recognized seminiferous tubules lacked the spermatogenic cells, the lumen of which was obliterated completely by Sertoli cells. (H&E stain X 250).

4. Discussion:-

Daily rhythms in behavioral and physiological processes are elicited by a set of connections of circadian clock; controlled by delivering circadian indicators to the brain and bodily organs. In mammals, at the top of circadian network is a main clock presents in the suprachiasmatic nuclei (SCN) of the hypothalamus, mainly rearranged by change in light. The nocturnal creation and release of melatonin by the pineal gland are firmly controlled by the SCN clock and inhibited by light contact [10 & 11].

Melatonin is a methoxyindole made and released principally by the pineal gland at night under standard environmental situations. The endogenous rhythm of melatonin production is entrained to the light/dark cycle, reaching its climax at darkness and minute level at light. Light is able to either suppress or synchronize melatonin secretion according to the light calendar [12]. The position of darkness in regulation of the physiology of neuroendocrine – reproductive association is still having a vast apprehension, in spite of decades of service in this circumstance [13, 14 & 15].

There were changes in the body weight of rats in all groups, though not all changes were statistically significant, this might be explained by the fact that darkness and its hormone termed melatonin cause effect on the overall body weight in rats [16 & 17]. This is probably because the food intake and/or bodily metabolism in rats are affected by melatonin [18 & 19].

The darkness affected the testicular weight in this work. The reason for that might be because the darkness directs the hypophysial-gonadal axis most probably through the high melatonin level which is well known to be increased during dark period [19]. Or in the course of the direct effect of melatonin through its receptors which are anticipated to be present in all biological tissues and cells [19, 20 & 21]. So, melatonin affected the testicular weight, and it is well known that testicular weight is primarily correlated to its physiological grade [22 & 23].

Looked upon testicular weight, seminiferous tubule wall thickness and lumen hole: there was an obvious optimistic effect of dark's hormone (melatonin) on these parameters which increased steadily with the raise in the length of the dark period, then after decreased in the last group. The grounds for this could be due to the idea that endogenous melatonin is known to be secreted in amount proportional to the length of exposure to darkness [1 & 3], and as it is well documented that melatonin exerts its achievement in a dose – dependent mode, being stimulating at standard level and injurious at higher level [24, 25 & 26]. The seminiferous tubule wall was thicker with more frequent appearance of mitotic figures observed in groups II, III and IV. These changes disappeared in group V. These findings might indicate the increase in number of spermatogenic cell lineage, which could be the consequence of endogenous melatonin's outcome on Sertoli cells of the spermatic tubules affecting them directly through melatonin receptors found in almost all tissues and cells [19, 20 & 21]. These cells secrete androgen binding protein (also called testosterone binding globulin) that increases testosterone concentration in the seminiferous tubules to stimulate spermatogenesis [5 & 6], or indirectly through melatonin's consequence on the pituitary gland affecting its secretion of gonadotropins [27], hence, promoting the process of spermatogenesis. The reverse process might be the cause of the decrease in the thickness of germinal epithelium and tubule lumen surface area in group V. The discussion for this could be due to the concept that melatonin is well designed nowadays to exert its physiologic action in a dose – dependent manner, being exciting at standard therapeutic level and destructive at higher intensity [24, 25 & 28].

The hormone inhibin, which is normally secreted by Sertoli cells, acts to inhibit the secretion of FSH by the pituitary under control of the hypothalamus [29]. Besides; other suggestion for the raise in number of spermatogenic cell lineage could be through repression of the hormone inhibin, by high amount of endogenous melatonin which could be the cause of speeding

spermatogenesis [30]; while in the last group the reverse process might probably be happening.

The number of points overlying the interstitial space was proportionate adversely to those points superimposed on the wall and lumen of the tubules. The reason for this could be due to the effect of endogenous melatonin either directly on the main cells of interstitial spaces termed the Leydig cells through melatonin receptors proposed to be present in approximately all living tissues and cells [19, 20 & 21], and / or indirectly by melatonin outcome on hypothalamic – hypophysial alignment holding back the secretion of LH which acts upon the Leydig cells affecting their activity and number [31 & 32]. The other scheme of explanation could be through rising stimulation of these leyding cells by melatonin inducing high amount of androgen which act through its negative feedback mechanism on the hypothalamus leading to inhibition of LH emission also [2, 31 & 32]. Multinucleated spermatogenic cells were seen only in group V, as these cells are seen only if there are harmful conditions to the testis [33].

The raise in numbers of apoptotic and pyknotic cells seen in groups II, III especially group IV, might be caused by the effect of melatonin on Sertoli cells to control the large number of spermatogenic cells competing for survival in a so called programmed cell death process which is very different from that which occurs as a direct result of severe damaging stimulus to cells, termed necrosis [5 & 6].

In group V: some areas showed severe fibrosis and necrosis with few retained spermatic tubules, this picture puts forward the extent of the highly negative effect of elevated level of endogenous melatonin, since in any damaging events to the testis an analogous histological spectacle would be seen [5, 6, 34, 34 & 35].

With exception to group V, Sertoli cells were not identified in all other groups, because it is well known that Sertoli cells are not distinguished by the routine histological paraffin section stained with hematoxylin and Eosin [5 & 6] ; therefore they were not studied in this work.

Dilatation of blood vessels was projected in all groups, which was comparative to the darkness length. The ground for that might be caused by the vasodilatation generation by melatonin [36 & 37].

The yellow-brown pigmentation perceived in the cytoplasm of Leydig cells is liopfusion pigmentation which is a sign of severe damaging circumstance applied to these cells [5, 6 & 34]. The epithelioid cells also appear merely in injurious conditions [6].

Our enduring studies on position of the well known neuro-hormone melatonin (whether endogenous or exogenous) on the hypothalamic – hypophysial – gonadal alliance, probably, could afford a new advance for dealing with many important testicular risky conditions causing sterility. Accordingly, such approaches might be extremely fruitful in the management of infertility – inducing events, in future. The location of melatonin in human well-being is still a milieu of controversy and a good subject of medical articles till now [3].

We think that pathophysiology of testicular hurt has not so far adequately studied in every stressful events to the testis. Melatonin is an influential scavenger of free radical, so supplementation of which, might be helpful in the prevention, or, at least, in the rescheduling of damaging measures on the testis [38].

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