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Gender-related Histological Changes in the Thymus Gland After Pinealectomy and Short-term Melatonin Treatment in Rats

Irfan Susko[1], Selma Alicelebic[2], Esad Cosovic[2], Maida Sahinovic[2], Dina Kapic[2], Samra Custovic[2], Visnja Muzika[2]

ABSTRACT

Objectives: The aim of this study was to investigate the effects of pinealectomy and melatonin treatment on the rat thymus gland characteristics, taking into consideration possible gender differences. **Materials and methods:** Thirty adult Wistar rats of both sexes were divided into three groups. Group C and group PX served as control groups and included sham-pinealectomized and pinealectomized animals that were treated with 10% ethanol solution (0,1ml/daily, subcutaneous). Animals from third group (group PXM) underwent pinealectomy and seven days after surgery started receiving melatonin dissolved in 10% ethanol solution (3mg/kg/daily, subcutaneous). All animals were treated for 4 weeks. **Results:** Volume density of the thymus cortex showed statistically significant ($p<0,05$) decrease while the volume density of the thymus medulla was increased in the pinealectomized compared to the sham-pinealectomized female rats. Numerical density of macrophages as well as the distribution of blood vessels showed no gender differences. The numerical density of lymphocytes was statistically significantly decreased in female in comparison to the male pinealectomized rats. Melatonin treatment was proved to cause reverse effects in the sense that the results from the melatonin treated group corresponded to the results obtained from the control group of animals. **Conclusion:** The results of this study suggest that the pinealectomy causes gender-related changes in the rat thymus. Short-term melatonin treatment showed reverse effect, equally in both sexes.

Keywords: rat, pineal gland, thymus gland, melatonin.

1. INTRODUCTION

Phylogenetically, the origin of the mammalian pineal gland is linked to an eyelike photoreceptive organ found in the lower vertebrates. The function of this gland is highly influenced by the stress signals through the sympathetic innervation (neurohumoral pathway). Pinealocytes produce melatonin, low-molecular-weight tryptophan derivate, in a diurnally fluctuating manner which in turn induce the circadian rhythm of physiological functions and behavior in all vertebrates. The retinohypothalamic tract leads the input generated in retina directly to the pineal gland enabling it to respond directly to the environmental cycle of light and darkness. Melatonin is known to positively affect the angiogenesis (1) act as the powerful free-radical scavenger (antioxidant) and important immunostimulator (increases the number of T-helper lymphocytes and NK-cells) that promotes the production of interleukins (2).

Melatonin shows direct and indirect effects. In vitro it directly affects sperm motility (3) and Leydig cells ability to produce steroids. Melatonin inhibits synthesis and releasing of endogenous ovulatory hormones by decreasing hypothalamic production of gonadotropin-releasing factors (4) via arginine-vasopressin system (5). Melatonin is capable to increase the transformation of progesterone into testosterone by enzyme activation, thus causing general inhibition of steroid biotransformation (5).

2. AIM

The aim of this study was to investigate the effects of pinealectomy and melatonin treatment on the rat thymus gland characteristics, taking into consideration possible gender differences.

3. MATERIALS AND METHODS

Animals. In this study we used thirty adult Wistar rats of both sexes, weighing 150–200g each. The animals were maintained in standardized laboratory conditions (12-hour light-dark cycle, temperature 23±2°C) with a standard diet and water provided ad libitum.

Experimental design. The animals were randomly divided into three groups where both sexes were equally distributed within each group. Group C (N=10) and group PX (N=10) served as control groups and included sham-pinealectomized and pinealectomized rats, respectively. Both groups were treated with 10% ethanol solution (0.1ml/daily, subcutaneous injection). Animals from the group PXM (N=10) underwent pinealectomy and seven days after surgery started receiving melatonin dissolved in 10% ethanol solution (3mg/kg/daily, subcutaneous injection). All chemicals were purchased from Caesar & Loretz GmbH and used without additional purification. The animals were treated for 4 weeks and then euthanized using ether anesthesia. The animals were handled according to approved institutional review board and the animal protection laws and guidelines.

Pinealectomy was done according to the Herbert's method, modified by Carter et al (6). Animals received intraperitoneal injection of anesthetic Nembutal in the dose of 5mg/100g BW (body weight). The back of the cranium was cut open just in front of the lambdoid suture so that the cerebral veins could be moved back and upward. The pineal gland was clearly exposed and removed. The ligation of sagittal sinus was avoided as an attempt to minimize possible brain damage and to preserve efficient venous drainage. After procedure, the bone flap was brought back and the wound was sealed. All the animals were monitored for possible postsurgical complications.

Histological analysis. In the end of experiment (24 hours after the last treatment), all animals were sacrificed using ether anesthesia. During the autopsy, previously done pinealectomy was verified for each animal. The thymus gland was removed, weighted and then fixed in Bouin solution. Paraffin-embedded tissue blocks were cut into 5 micrometers thick sections and stained with the following methods: haematoxylin and eosin (HE), periodic acid - Schiff (PAS) and Gomori-Bergmann silver impregnation staining technique (7).

Stereological analysis was performed using light microscopy and Weibel test frames C-64, B-25 as well as the multipurpose M-42 test system with an ocular micrometer 1:100. The number of hits was determined in the cortex, medulla and interstitial space for all the chosen test-points of the thymus gland. Following the point counting, the volume density (Vv) was estimated for the tissue volume unit. Combining test frames with an objective magnification x 60, it was possible to determine the number of hits and numerical density (Nv) of lymphocytes and epitheloid cells. Lymphocytes were clas-

	Male			Female		
	N	Thymus gland mass (mg)*	Relative thymus mass mg/100g*	N	Thymus gland mass (mg)	Relative thymus mass mg/100g
C	5	224.71±9.48	154.72±9.81	5	265.43±11.21	164.72±5.01
PX	5	216.42±7.43	156.64±10.23	5	218.35±10.05**	150.34±12.31
PXM	5	198.33±8.76	142.45±11.42	5	260.33±9.76	168.41±12.22

Table 1. Absolute and relative thymus gland mass of male and female rats. C- control group, PX – pinealectomy, PXM – pinealectomy + melatonin. * The results are expressed as mean±standard deviation. ** Statistically significant decrease in comparison to the group I, p<0.001

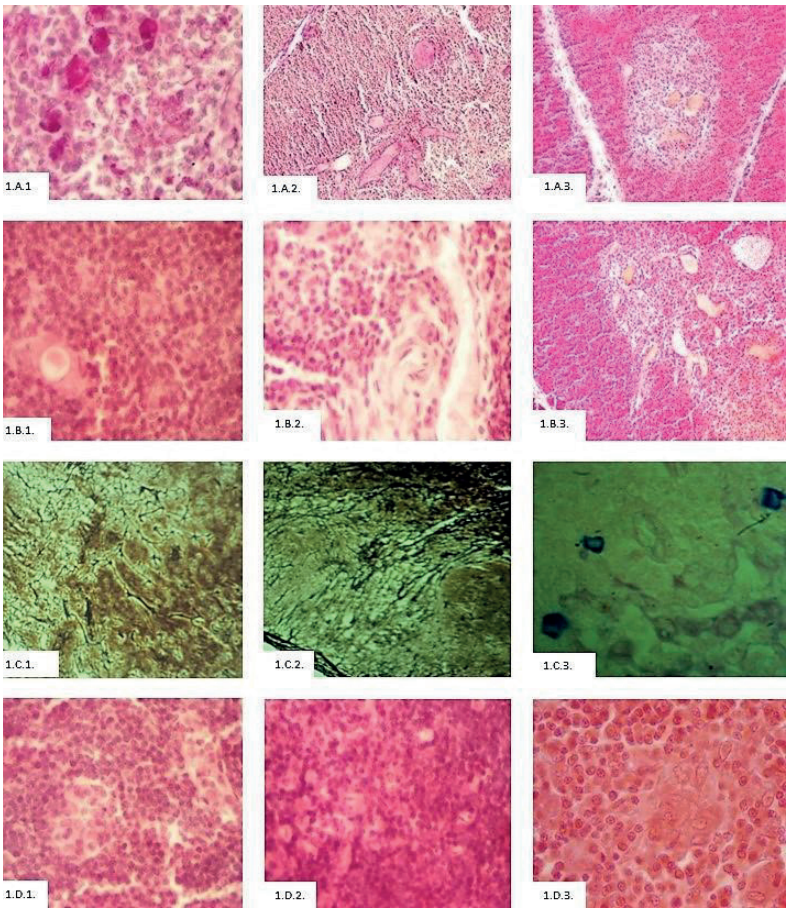

Figure 1. Depleted thymus cortex of the pinealectomized female rat with obviously increased number of macrophages (1.A.1.) and large blood vessels (1.A.2.) in the corticomedullary boundary. Medulary area of the male pinealectomized rat shows prominent connective tissue with blood vessels (1.A.3.). Epitheloid cells forming tubular structures with content (1.B.1.) or whirl-like organisations (1.B.2.) as found in thymus of pinealectomized females. Thymical medulla with enhanced angiogenesis and blood vessels penetrating through the cotrico-medullary area (1.B.3.). Reticular stroma of the pinealectomized untreated (1.C.1.) and pinealectomized treated animals (1.C.2.). Mast cells in the thymic cortex characterised by smaller cell body and rather large nucleus with homogenous material (1.C.3.). Melatonin treatment stimulates lymphogenesis which is visible as zone of tightly packed lymphocytes and lymphocytes-strings surrounding depleted areas (1.D.1.). Epitheloid cells appear to be hypertrophic in thymic cortex (1.D.2.). Epitheloid cells forming islands in the thymic medulla (1.D.3.).

Vv mm0	Male			Female		
	C	PX	PXM	C	PX	PXM
Cortex	0.664± 0.05	0.655±0.03	0.659±0.06	0.634± 0.05	0.508±0.03	0.523±0.06
Medulla	0.310±0.04	0.293±0.02	0.301±0.04	0.362±0.04	0.373±0.02	0.441±0.04**
Interstitial space	0.026±0.002	0.052±0.01	0.04±0.02*	0.004±0.001	0.119±0.01	0.036±0.02***

Table 2. Gender-related differences in the volume density of the thymus gland compartments in the control and experimental group of animals. C- control group, PX – pinealectomy, PXM – pinealectomy + melatonin. *Statistically significant increase in comparison to the group I–males, p<0.05 ** Statistically significant increase in comparison to the group I–females, p<0.01 ***Statistically significant decrease in comparison to the group I–females, p<0.05

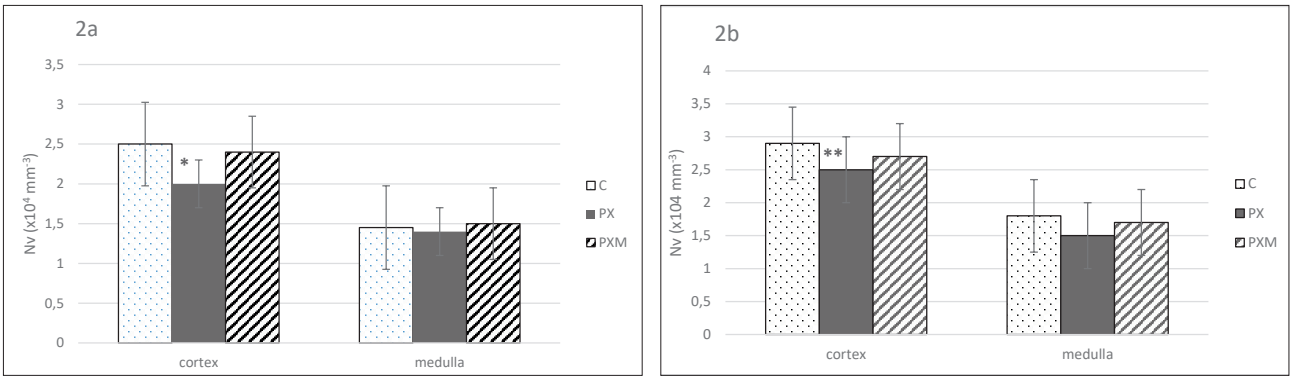

Figure 2. Numerical density of lymphocytes in cortex and medulla of thymus gland in male (2a) and female (2b) rats. C- control group, PX – pinealectomy, PXM – pinealectomy + melatonin.

sified into three classes regarding the nucleus diameter: 4, 6 and 8 micrometers. Further, distribution of all three lymphocyte classes was determined in the cortical and medullar area of thymus gland. Numerical density was counted according to the Kalisnik method (8).

Numerical density of epitheloid cells and mast cells was estimated using the Weibel-Gomez formula.

Statistical analysis. All results were expressed as mean ± standard deviation. Comparison between groups was determined using Student's t-test and variance analysis. P-value less than 0.05 and 0.01 was considered statistically significant. Analysis was performed using MS Excel 2016 for Windows.

4. RESULTS

Gross analysis. The mean thymus mass in the group PX (pinealectomized rats) was decreased in comparison to the group C (sham-pinealectomy) and reduction was statistically significant for the female rats (Table 1). The mean thymus mass was found to be significantly decreased in the untreated (group PX) in comparison to the treated pinealectomized rats (group PXM). The difference between pinealectomized treated (group PXM) and sham-pinealectomized (PX) animals was insignificant. C- control group, PX - pinealectomy, PXM - pinealectomy + melatonin.

Qualitative histological analysis. Qualitative histological analysis revealed that fatty degeneration of the thymus was generally more frequent finding in the female rats. Pinealectomy was related to the lymphocyte depletion as well as the loss of the clear distinction of the cortico-medullary boundary in both sexes. Increased number of macrophages and larger blood vessels were present in the thymus cortico-medullary boundary of female rats (Figure 1.A. 1-3).

Comparison of the serial slides of thymus revealed no gender-related differences in the cortex-medulla ratio even though very narrow, ring-like cortex was more frequent finding in females.

Prominent findings in the thymus of pinealectomized female rats were the whirl-like organizations of epitheloid cells, localized especially around large blood vessels. In treated female rats, epitheloid cells were often found forming tubular structures filled with clumped material (Figure 1.B. 1-3).

Thymic stroma of the treated animals appeared coarser than the stroma of untreated pinealectomized animals. Gender-related changes in the appearance of stromal tissue were inconspicuous. Mast cells were found often in the cortical region in close proximity of the macrophages. These cells appeared smaller, irregularly-shaped and filled with homogeneous material. Frequent finding was degranulation in the nearby septal connective tissue (Figure 1.C. 1-3).

Normal structure of the thymus was restored in the treated group and characterized by increased density of lymphocytes as well as signs of stimulation and proliferation of epitheloid cells (Figure 1.D. 1-3).

Quantitative histological analysis - stereology. Volume density of thymus cortex, medulla and stroma (interstitial space) is found to be statistically significantly changed in pinealectomized in comparison to sham-pinealectomized animals, more prominently in females.

Volume density in the thymus medulla of the treated females was significantly increased in comparison to the control group (C). At the same time, in treated males there was an increase in the amount of the interstitium (Table 2). Numerical density of the lymphocytes and their size-related distribution were changed in a different manner in males and females after the pinealec-

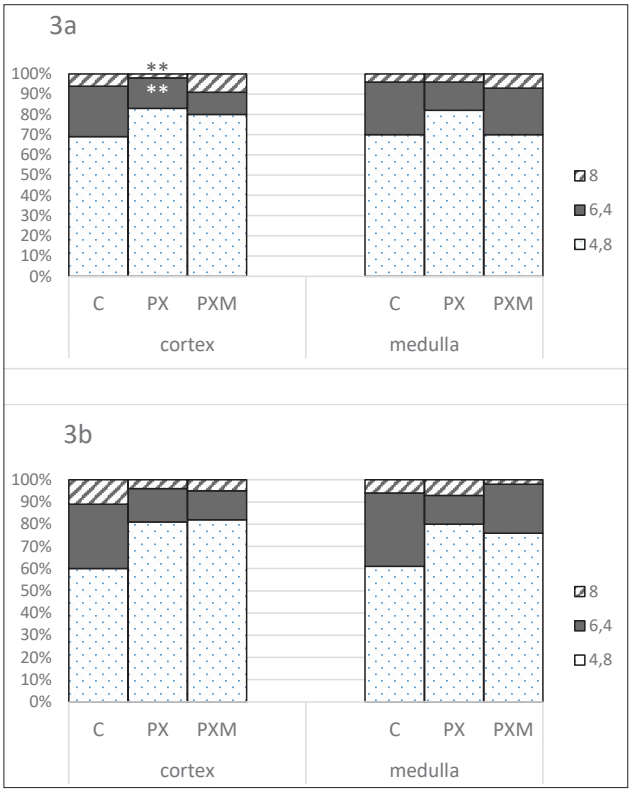

Figure 3. Lymphocyte size distribution in cortex and medulla of male (3a) and female (3b) rats.C- control group, PX – pinealectomy, PXM – pinealectomy + melatonin.

tomy and melatonin treatment. Decrease of density of the lymphocytes in thymic cortex was evident after the pinealectomy in male rats, while this change was not statistically significant in thymic medulla. After treatment with melatonin, numerical density of lymphocytes in the cortex reached the one in the control group. Melatonin treatment showed no significant effect in the medulla (Figure 2a).

In pinealectomized female rats, numerical density of the cortical lymphocytes was also decreased but melatonin treatment restored the values that were in range of the values of control group of animals. In thymic medulla of both, pinealectomized and melatonin group of female rats, no significant alterations of the lymphocyte numerical density were observed (Figure 2b).

According to the lymphocyte size distribution, it is evident that pinealectomy significantly, almost at half, decreased the number of large and medium-sized lymphocytes in thymic cortex of male rats. Melatonin administration increased the percentage of large lymphocytes in cortex as well as in medulla while the increase of medium-sized lymphocytes count was more significant (Figure 3a).

Pinealectomy in thymic cortex of female rats caused similar changes: decrease in number of large and medium-sized lymphocytes that were generally more resistant to melatonin treatment. In thymic medulla of female rats, the reduction in number of medium-sized lymphocytes was attenuated with melatonin treatment. These effects were comparable to the findings in the control group of animals (Figure 3b).

5. DISCUSSION

The present study shows that pinealectomy causes significant reduction of the thymus weight in female rats. Generally, thymus weight was not significantly affected by exogenous melatonin treatment in both sexes.

Histological analysis showed notable reduction of the thymic cortex volume density in pinealectomized female rats as well as frequent finding of fatty replacement. Decreased density of cortical lymphocytes and loss of distinct cortico-medullary boundary was observed in both sexes. The number of macrophages and large blood vessels in the pinealectomized female rats was significantly increased with apparent finding of „whirl-like" structures made of proliferated epitheloid cells localized around those blood vessels.

Treatment with exogenous melatonin caused hyperplasia of mast cells with frequent findings of the degranulation around chain-like clusters of macrophages. Administration of melatonin in female rats was accompanied with transformation of epitheloid clusters into tubular structures, frequently filled with clumped content. Also, treatment with melatonin in both sexes was accompanied with a coarser stroma of reticular fibers.

Quantitative analysis showed statistically significant increase of volume density in the thymus medulla of female rats after treatment with melatonin, while in male rats there was a significant increase in the amount of thymic stroma.

Numerical density (Nv) of lymphocytes was decreased in the thymic cortex in the pinealectomized rats of both sexes. Treatment with melatonin increased a density of the cortical lymphocytes up to the values found in the control group of animals of both sexes, while density of the medullary lymphocytes did not show significant alterations.

Distribution of large lymphocytes in the thymic cortex of male rats was significantly decreased, while relative proportion between small and medium-sized lymphocytes remained unaltered. Relative proportion among medullary lymphocytes did not change. After treatment with melatonin, the number of large lymphocytes significantly increased in the thymic cortex.

The same alterations in relative proportions of lymphocytes were detected in the thymic cortex of the female rats, while the reduction of medium-sized lymphocytes was evident in medulla. Treatment with melatonin ameliorated this reduction to the level of control group of animals.

There are numerous studies investigating the effects of pinealectomy on the animals' general state and immune system. Therefore, it was shown that neonatal pinealectomy disturbs the normal development of the animals (chicken) and induces the general atrophy, which led to the increased mortality rate. Furthermore, changes in the thymus were described as narrowed cortical area and consequently widened medullary region (9). Nevertheless, loss of the lymphocytes and increased number of structures similar to the Hassal bodies with appearance of the cystic formations was evident.

Gender-related reactive changes in the thymus gland that were found in this study are in accordance with our previous findings in rats after long-term melatonin treatment (10). It was already shown that the pineal gland produces most of its neuroendocrine effects indirectly by affecting the other endocrine glands, primarily suprarenal and gonads (9). After the pinealectomy, antigonadotropic activity of the pineal gland was lost and consequential hyperactivity of the female gonads and suprarenal gland was induced. Therefore, ovarian steroids caused the hypertrophy of the suprarenal gland thus affecting the epitheloid compartment of the female thymus gland. The endocrine system in female rats is more sensitive to the altered environmental factors (11) and epitheloid cells in the female thymus have a specific receptors for the estrogen (12) which stimulates them (13). These facts can explain the gender-dependent reaction of the epitheloid cells in the pinealectomized animals. Poor stimulation of the thymic-epitheloid cells in the pinealectomized male rats is in accordance with the absence of the epitheloid proliferation around thymic testosterone implants because of the testosterone suppressive effects on epitheloid cells (13). The importance of the estrogen regulation of the glycoprotein accumulation was already proved by Bennett et al. (14). These findings are in accordance with the PAS-positive content in the tubular structures of the pinealectomized females in our study.

The macrophages showed increased activity and were more numerous in the cortico-medullary area of the thymus of pinealectomized animals which can be explained by their enrollment in the formation of the immunocompetent lymphocytes and their migration patterns (15). Thymical phagocytes produce interleukins and PGE2 (16) thus controlling the proliferation of the lymphocytes. Pfeifer and Patterson (17) determined that thymus maintains the potency of the lymphokine producing T-lymphocytes which activate the macrophages. Increased production of these lymphokines is induced by estrogen, thus making the thymic activation of the T-lymphocytes and stimulation of macrophages in the pinealectomized animals questionable.

The mast cells distribution showed sexual dimorphism, reflecting in their significantly increased number in the Harderian gland of the female rats compared to the male rats (18). The main content of the granules in mast cells is serotonin, which acts as a vasoconstrictor and reduces permeability of the blood capillaries. This can explain preserved density of the lymphocytes in the thymus medulla after the pinealectomy. Catecholamines released by the mast cells affect the noradrenergic fibers in the thymus, thus modulating the activity and maturation of lymphocytes (19).

Suppressive effect of the pinealectomy on a lymphogenesis (reduction in the cell number and their volume) was described by Oner et al (20). Pearce et al. (21) found that the involutive changes in the thymic lymphoid population in the pinealectomized animals were result of an increased activity of the estrogen and androgen steroids. These hormones binds to the specific receptors with the same affinity in both sexes (21). Sex steroids prevent lymphocyte mitosis by blocking the calcium influx and by inhibiting the cAMP synthesis (22).

The melatonin itself is an important transmitter of a photoperiodic information, it has a significant antioxidant properties and acts as an immunomodulator (23). Melatonin is synthesized in the vertebrate retina (photoreceptors cones) and has local neuromodulatory properties as a free radical scavenger in the photoreceptors. Pineal melatonin controls the daily rhythm of circulating melatonin and acts a signal of nighttime that controls many other biological rhythms (24). Light from the retina goes to suprachiasmatic nucleus (endogenous circadian oscillator) and promotes gene expression that control the circadian output signals from the suprachiasmatic nuclei. There are three main components of the circadian system in mammals: lateral eyes, hypothalamic suprachiasmatic nucleus and the pineal gland. Suprachiasmatic nucleus adjusts with the environmental light conditions by retinal input. Received informations are distributed throughout the body at a certain time. Output signals from the suprachiasmatic nucleus affects the pineal gland by periodic release of noradrenalin from sympathetic fibers during the night (25, 26). It binds to the nuclear receptors, which belong to the family of retinoic acid receptors and resides in the immune cells (lymphocytes). Because of that, its activity in lymphoid tissue recovery after the pinealectomy is understandable (27). The actions of melatonin are ambiguous: direct (receptor-mediated or receptor-independent (28) or indirect: through stimulation of the immune system. The latter affects the humoral and cell-mediated immune responses (29) and regulate a lifespan of the leukocytes by controlling the apoptotic events. These processes cooperate with degranulation of mast cells (30). Melatonin stimulates the production of macrophages, NK cells and phagocytes (31) which explains the increased number of macrophages found in the thymus of melatonin-treated pinealectomized female rats in our study. Our findings of the enhanced vascularization in thymic medulla is in accordance with the findings of Soybir et al. (1) that melatonin has positive effects on angiogenesis.

Although reactivation of endocrine activity of thymus in older animals could be achieved with different endocrine and nutritional manipulations (e.g. melatonin, growth hormone, gonadotropin-releasing hormone and zinc supplements) there is no definitive conclusion about their effects on thymic epitheloid component (32). Zinc (Zn) reactivates Zn-dependent enzymes for cell proliferation and apoptosis and thymulin, a zinc-dependent thymical hormone. Mahmoud et al. (33) found considerable histological alteration of the thymus gland in male rats that were kept in the constant darkness (epitheloid cells and lymphocytes are stimulated in both, cortex and medulla and perivascular spaces are widened) but no data is provided for the female rats.

6. CONCLUSION

We conclude that exogenous melatonin predominantly affects the recovery of lymphoid tissue and increases

the amount of thymical stroma with the cells of innate immunity (macrophages, dendritic cells). This is in accordance with findings of Calvo JR et al (34).

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