

Melatonin supplementation restores cellular proliferation and DNA synthesis in the splenic and thymic lymphocytes of old rats

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Abstract

OBJECTIVES: In this study we investigated the effect of melatonin treatment on the proliferative activity, the rate of DNA synthesis and the histopathological changes of splenic and thymic lymphocytes in old rats.

METHODS: Two subgroups of old rats (25-months-old) were used in this study. One subgroup was given melatonin in the drinking water (250-300 µg/day/rat) for 3 months while the second subgroup was given water containing diluent. A third group consisted of young rats (3-months-old) which served as an additional control.

RESULTS: A ³H-thymidine autoradiographic investigation showed a reduction in both the proliferative activity and the rate of DNA synthesis in splenic and thymic lymphocytes in old rats. In addition, light and electron microscopy showed severe histopathological changes in these cells from diluent-treated old rats. Melatonin administration increased the proliferative activity and the rate of DNA synthesis in the lymphocytes of both the spleen and thymus of the old animals. Also, histopathological changes were partially reversed by melatonin treatment with the tissues appearing similar to those in the young rats.

CONCLUSION: The stimulation of the lymphocyte activity by melatonin is a beneficial response, especially in old rats, since aging results in an inhibition in lymphocytic functions.

Introduction

A considerable amount of evidence has demonstrated that the pineal gland, via its secretory product melatonin, enhances immune function either at the central or peripheral level [1–3]. The administration of pineal extracts or melatonin induces thymic hyperplasia, increases antibody responses, promotes the proliferative response to ConA, and increases antigen presentation by macrophages [4,5]. Conversely, surgical or pharmacological pinealectomy induces thymic involution, depresses hormonal and cell-mediated immune responses, and reduces interleukin-2 (IL-2) production [4,6–8] while melatonin administration reduces the loss of thymocytes [9].

In mice, cells derived from the spleen of pinealectomized animals display reduced natural killer (NK) cell activity and IL-2 production, responses that are restored by melatonin administration [8]. The immunoenhancing effects of melatonin may be particularly relevant to old age since plasma melatonin levels diminish with advanced age in all mammals where it has been investigated, including the human [10–13], and typically melatonin administration results in a significant restoration of immune parameters normally depressed in aged organisms [14–16]. It has been reported, for example, that melatonin treatment reverses age-related thymic involution, reduces thymic endocrine activity and T-lymphocyte numbers, as well as overcoming impaired antibody responses, T helper activity and IL-2 production in old animals [17,18]. Provinciali et al [19] demonstrated that melatonin treatment prevents age-related thymic involution through regulation of thymocyte apoptosis.

The destructive consequences of endogenously-generated free radicals are usually estimated in terms of damage to macromolecules, most notably, lipids, proteins and DNA. The gradual accumulation of the resulting damaged products throughout a lifetime has been proposed to be consequential in the processes of aging and age-related diseases [20–22]. Molecules and/or processes that neutralize free radicals or prevent their formation are referred to as antioxidants. Melatonin is a highly effective antioxidant [23–25]. Due to the age-associated drop in pineal and blood melatonin concentrations, old individuals in the population are generally considered to be relatively melatonin-deficient [13]. Based on the data summarized above, the drop in melatonin in old animals may help to explain, in part, the reduction in immune function.

Considering this, the aim of this work was to investigate the efficacy of melatonin treatment in promoting the proliferative activity and the rate of DNA synthesis as well as overcoming the histopathological changes in splenic and thymic lymphocytes in old rats.

Material and methods

Animals

Male Sprague-Dawley rats with the initial ages of 3 (young) or 25 (senescent) months were used in this experiment. There were 8 young rats and 16 old animals. The animals were housed conventionally in cages (2–3 rats per cage) and fed with standard food and tap water ad libitum. The animals were maintained on a 12 h light/12 h dark cycle at constant temperature ($25\pm 2^\circ\text{C}$). An automatic timer controlled dark and light exposure with lights off daily from 19.00 h to 07.00 h. The care and treatment of the animals was approved and performed according to the guidelines of the University of Assiut.

Chemicals

Chromatographically pure melatonin was a gift from Helsinn Chemicals SA (Biasca Switzerland). Tritiated thymidine ($[^3\text{H}]\text{TdR}$) was purchased from New England Nuclear (Boston, MA). Kodak NTB₂ emulsion, Kodak D-19 developer and Kodak fixer were purchased from Eastman Kodak (Rochester, New York). All other chemicals were of the highest quality available.

Experimental design and procedures

The rats were divided into three groups. The first group (3-mon-old) served as young controls. The senescent (25-mon-old) rats were divided into 2 subgroups. Subgroup 1 served as old controls and received tap water containing 0.001% ethanol for 3 months. Subgroup 2 was given melatonin, as follows, for three months. 10 mg of melatonin were dissolved in ethanol and diluted to 400 ml with tap water. The melatonin solution was prepared fresh daily. The final concentration of melatonin in the drinking tap water was 25 $\mu\text{g}/\text{ml}$. Melatonin was present in the drinking water from 18.00 to 08.00 h daily (from 1 h before lights off to 1 h after lights on). Melatonin was available for 3 mon using this regimen. Water intake was monitored at weekly intervals. Water intake ranged from 10–12 ml/night/rat (roughly 250–300 μg melatonin/night/rat).

Autoradiography

After the 3 mon treatment period, young and old rats were killed at which time they were 6 and 28 mon of age, respectively. Before decapitation, all animals received a subcutaneous injection of 1 $\mu\text{Ci}/\text{gm}$ body weight of $[^3\text{H}]\text{TdR}$ and they were killed 2 hours later. Portions of the spleen and thymus were fixed in 10% neutral buffered formalin and 5 μm thick paraffin sections were prepared. The deparaffinized sections were dipped in Kodak NTB₂ emulsion (diluted 1:1 with distilled water) and kept for 18 days in the dark at 4 $^\circ\text{C}$. Thereafter, they were developed in Kodak D-19 developer for 3 min and fixed in Kodak fixer for 5 min at 15 $^\circ\text{C}$. A cell was scored as being labelled when it showed 5 or more grains over its nucleus; the individual who scored the slides was blind to the treatment and to the age of the animal from which the tissues were collected.

Quantitation of autoradiographs

A total of 12,000 labelled and unlabelled lymphocytes were counted in each spleen and thymus. The ^3H -labelling index (LI) was expressed as a percentage of the total lymphocytes counted. The grain count per labelled nucleus (GC/N) was evaluated by dividing the total number of silver grains over the labelled nuclei by the total number of labelled cells. The LI and GC/N represent the kinetics of proliferation and the rate of DNA synthesis, respectively.

Histopathology and Electron Microscopy

For histopathological examination, portions of the spleen and thymus were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. The sections were stained with H&E. For electron microscopy, other portions of the spleen and thymus were fixed in 2.5% glutaraldehyde in cacodylate buffer. The specimens were washed in cacodylate buffer (0.1 M, pH 7.2) for 1–3 hours and then post-fixed in 1% osmium tetroxide for 2 h. The specimens were placed in propylene oxide for 60 min, then in pure epon 812 and incubated in a special polymerization incubator (one day at 37°C, second day at 45°C and then three days at 60°C). Semithin sections were obtained and stained with toluidine blue and examined using a light microscope. Representative fields of semithin sections were selected. Ultrathin sections were mounted on copper grids and stained with uranyl acetate, lead citrate and investigated with TEM.

Statistics

The data are expressed as means \pm standard errors (SEM). Differences between groups were determined using an ANOVA followed by the Student-Newman-Keuls *t*-test. The level of significance was accepted with $P < 0.05$. The percentage stimulation (S%) or inhibition

(I%) in the mean values of LI and GC/N were calculated as follows:

$$1\% \frac{\text{Mean young control value} - \text{mean old control value}}{\text{mean young control value}} \times 100$$

$$S\% \frac{\text{Mean old melatonin value} - \text{mean old control value}}{\text{mean old melatonin value}} \times 100$$

Results

The distribution of labelled lymphocytes and the density of grains over the labelled nuclei in the spleen of old rats not given melatonin are shown in *Figure 1-A*. In rats which given melatonin daily in drinking water, the number of labelled lymphocytes and grains over labelled nuclei were increased versus old control rats as shown in *Figure 1-B*. The quantitative results are presented in Table 1. These results reveal a significant reduction in the mean values of LI and GC/N ($P < 0.01$) in old control rats versus young control animals. In old rats, both the LI and the GC/N were lower by 37% and 40% respectively, when compared to those in young control rats. In old rats which treated with melatonin for 3 months, there was an increase $P < 0.01$ in the mean values of GC/N versus old non-melatonin treated rats although the increase in the LI was nonsignificant. There was 12% and 20% stimulation in proliferative activity and the rate of DNA synthesis, respectively in old melatonin-treated rats versus old non-treated animals.

In the thymus, the distribution of labelled lymphocytes and the density of grains over labelled nuclei of old control rats are shown in *Figure 1-C*. In old melatonin-treated rats there was an increase in both the number of labelled lymphocytes and the grains over

Table 1. Mean ^3H -labelling indices (\pm SEM), mean grain count per labelled nucleus (\pm SEM) and inhibition or stimulation (S) (%) in the splenic lymphocytes as a consequence of age or melatonin treatment.

Group	^3H -labelling index	Inhibition or stimulation	Grain count per labelled nucleus	Inhibition or stimulation
Young control	7.64 \pm 0.31	–	23.76 \pm 0.85	–
Old control	4.83 \pm 0.26*	I = 37%	14.19 \pm 0.60*	I = 40%
Old melatonin	5.46 \pm 0.20	S = 12%	17.71 \pm 0.75**	S = 20%

* $P < 0.01$ vs young control; ** $P < 0.01$ vs old control.

Table 2. Mean ^3H -labelling indices (\pm SEM) mean grain count per labelled nuclei (\pm SEM) and inhibition (I) or stimulation (%) in the thymic lymphocytes as a function of age or melatonin treatment.

Group	Mean ^3H -labelling index	Inhibition or stimulation	Mean grain count per labelled nucleus	Inhibition or stimulation
Young control	1.74 \pm 0.17		12.44 \pm 0.63	
Old control	0.79 \pm 0.11*	I = 55%	7.51 \pm 0.42*	I = 40%
Old melatonin	1.01 \pm 0.11**	S = 22%	9.28 \pm 0.52**	S = 19%

* $P < 0.01$ vs young control; ** $P < 0.01$ vs old control.

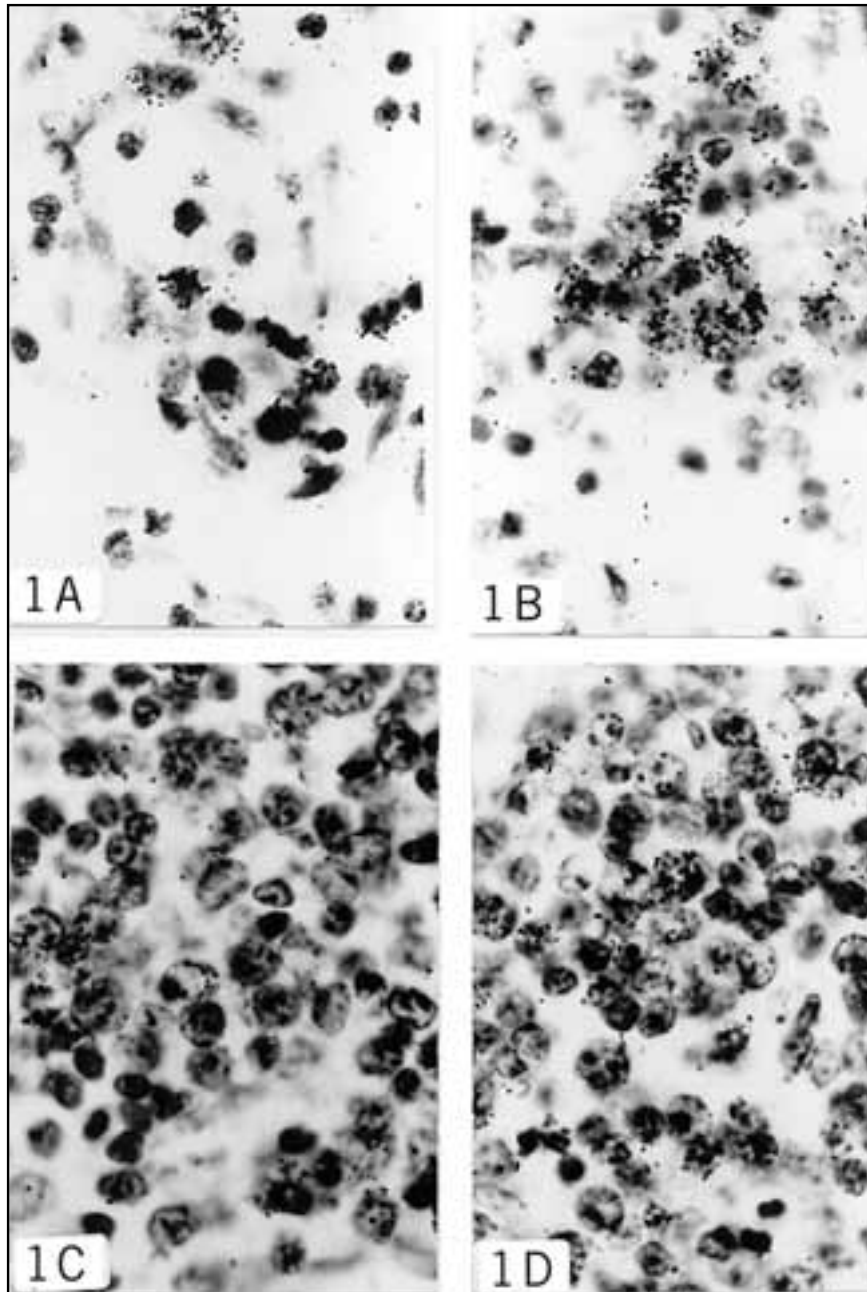


Figure 1, to the left

1A: A section of the spleen of old rat showing the distribution of labelled lymphocytes and the density of grains over the labelled nuclei. H & E (X 1000).

1B: A section of the spleen of old rat treated with melatonin showing the distribution of labelled lymphocytes and the density of grains over the labelled nuclei. H & E (X 1000).

1C: A section of the thymus gland of old rat showing the distribution of labelled lymphocytes and the density of grains over the labelled nuclei. H & E (X 1000).

1D: A section of the thymus gland of old rat treated with melatonin showing the distribution of labelled lymphocytes and the density of grains over the labelled nuclei. H & E (X 1000).

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Figure 2, to the right

Sections of spleen of old rats showing:

2A: Exuhaustion of lymphocytic elements in both white and red pulp with extramedullary hematopoiesis (arrows) in the splenic sinuses.

2B: Increase number of megakaryocytic cells (arrows) and dilatation of splenic sinuses.

2C: Normal population of lymphocytes in both white and red pulp with presence of few megakaryocytes (arrows) in melatonin-treated old rat. H & E (X100).

Figure 3, to the right

Sections of thymus of old rats showing:

3A: Proliferating epithelial cords forming cyst (C) filled with pink homogenous material.

3B: Sever thymic atrophy with appearance of fibrovascular tissues.

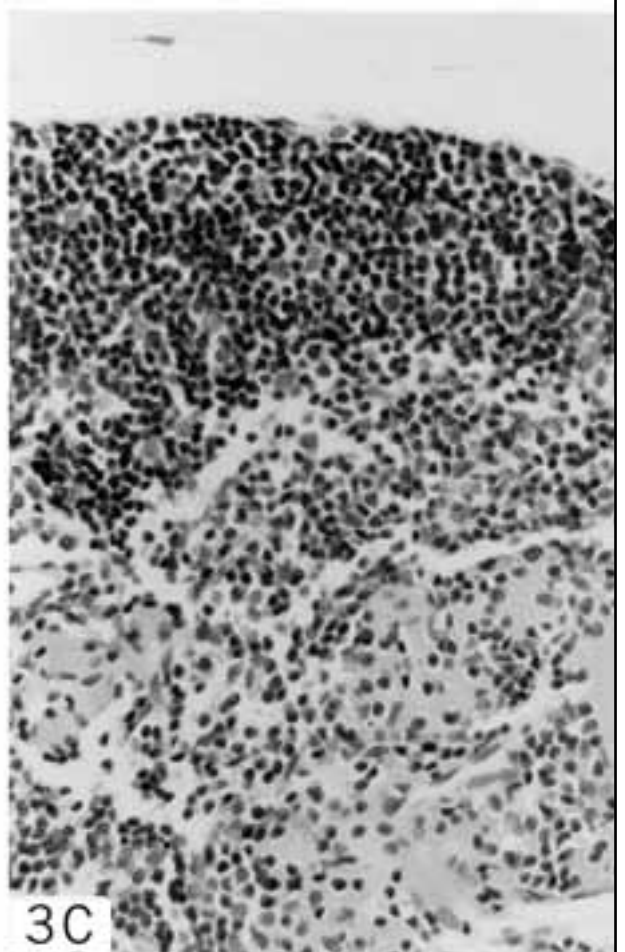
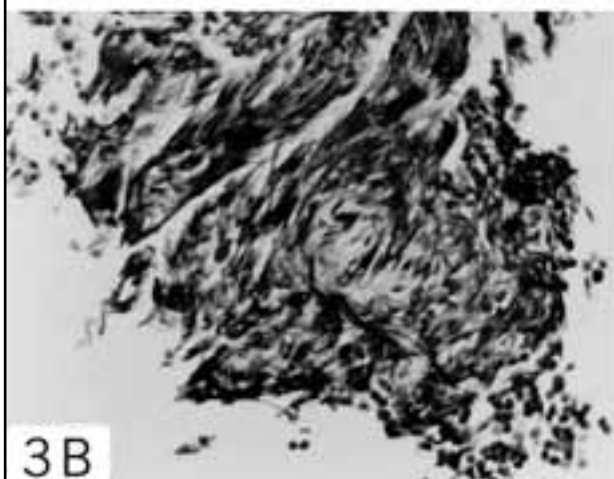
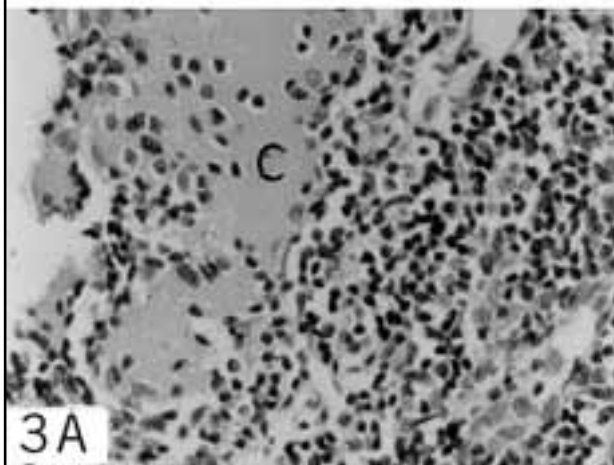
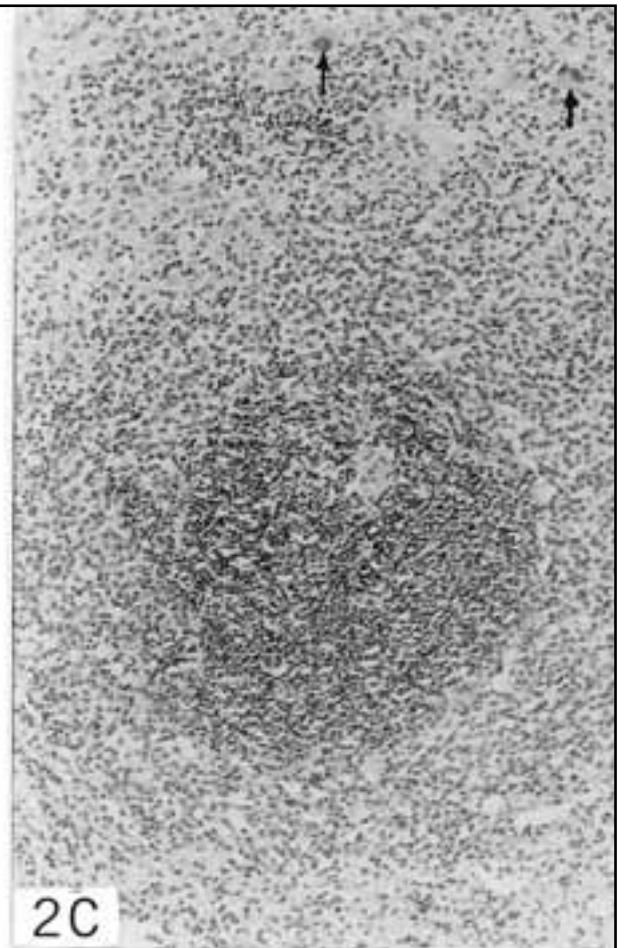
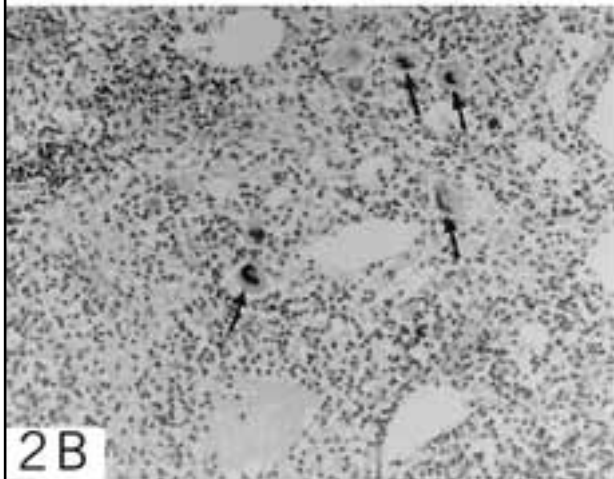
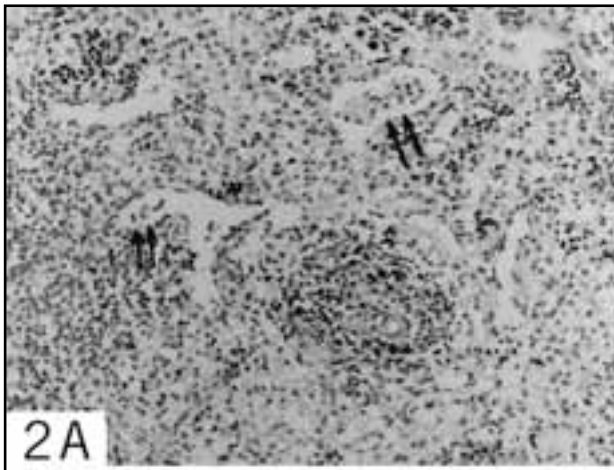
3C: Normal cortical structure with proliferation of epithelial cells and cystic formation in the medullary part in melatonin-treated old rat. H & E (X250).

labelled nuclei (*Figure 1-D*). The quantitative results are presented in Table 2. There was a significant reduction in the mean values of LI and GC/N ($P < 0.01$) of old control rats versus young animals. The inhibition in proliferative activity and the rate of DNA synthesis in old rats was 55% and 40%, respectively, when compared to those in young rats. In old rats which were treated with melatonin, there was an increase in the mean values of LI and GC/N versus old control rats. Statistically, the increase was significant ($P < 0.01$) for both LI (22%) and GC/N (19%).

Histopathological examination showed a loss of lymphoid elements in both the white and red pulp in the spleen of old rats (*Figure 2A*), also apparent was extramedullary hematopoiesis in the spleen of these animals. The hematopoietic cells were located in dilated splenic sinuses in the red pulp. An increased number of megakaryocytes was observed in the red pulp (*Figure*

2B). Hemosidrosis and accumulation of hemosidrin pigments in sidrophage cells was infrequently seen. In the spleen of old rats which were treated with melatonin, there was a relatively restoration of lymphocytic elements in both the white and red pulp. However, extramedullary hematopoiesis and megakaryocytic activity were reduced (*Figure 2C*).

In the thymus gland of old rats there was a loss of cortical lymphocytes with apparent proliferation of epithelial cells especially at the medulla when compared with young controls. The epithelial cells formed cords with cystic dilatation containing pink homogenous material (*Figure 3A*). In some cases, the majority of the gland appeared atrophic with the formation of fibrovascular tissue (*Figure 3B*). Occasional small nests or foci of lymphocytes were apparent. In the melatonin-treated rats, the gland contains numerous cortical lymphocytes and the medulla showed an apparent prolifer-



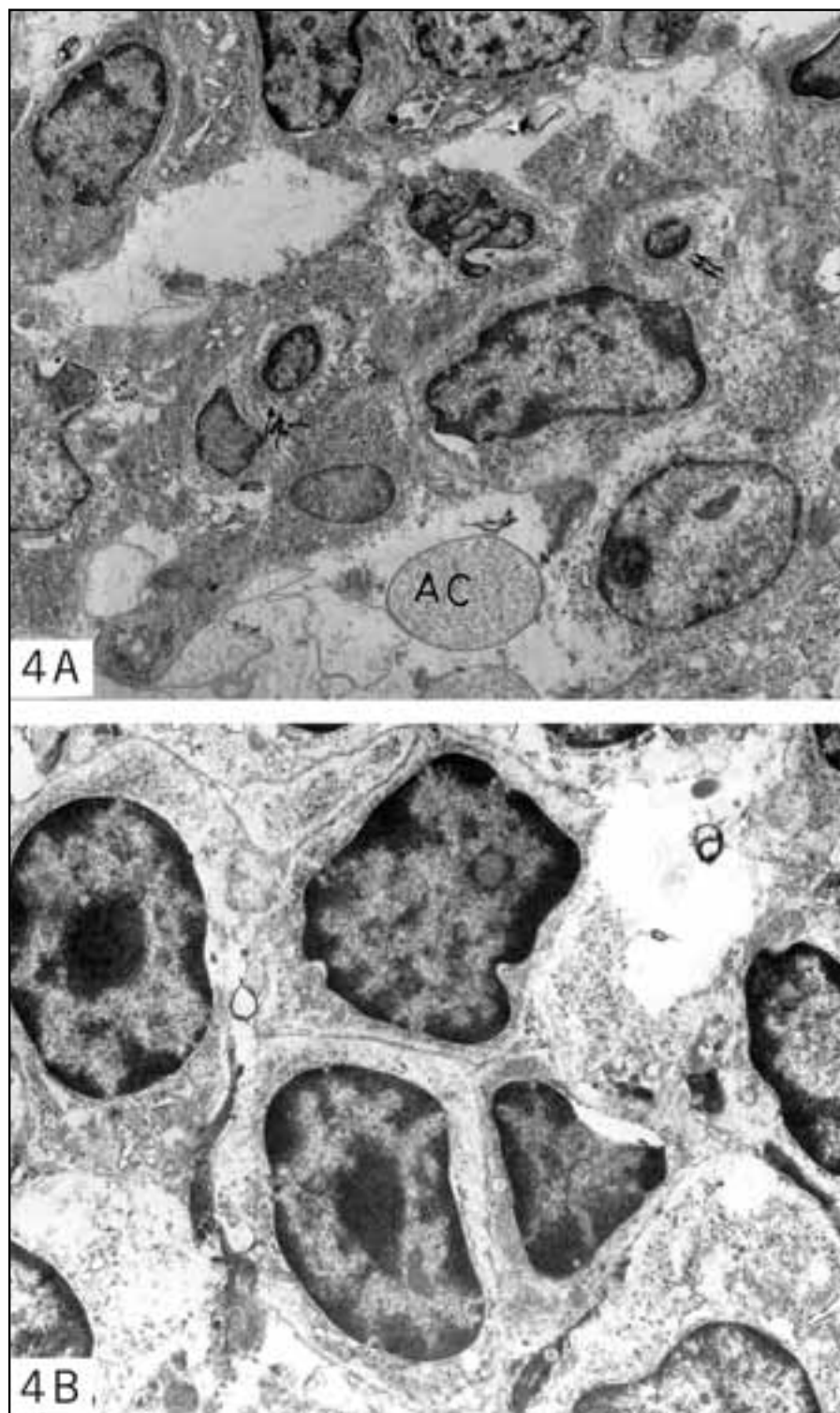


Figure 4.

Transmission electron photomicrograph of spleen:

4A: Old rat showing many apoptotic lymphocytic cells; some showed condensation of both nuclear chromatin and cytoplasmic matrix (arrows) and others appeared as a cell membrane contain remnants of cytoplasmic organells (AC).

4B: Old rat treated with melatonin showing only vacuolar degeneration in the lymphocytic cytoplasm, the nuclei are more or less normal. Uranyl acetate, lead citrate (X5000).

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eration of epithelial structures with a lumen contained pink homogenous material (*Figure 3C*). Fibrovascular tissues were not seen in this group.

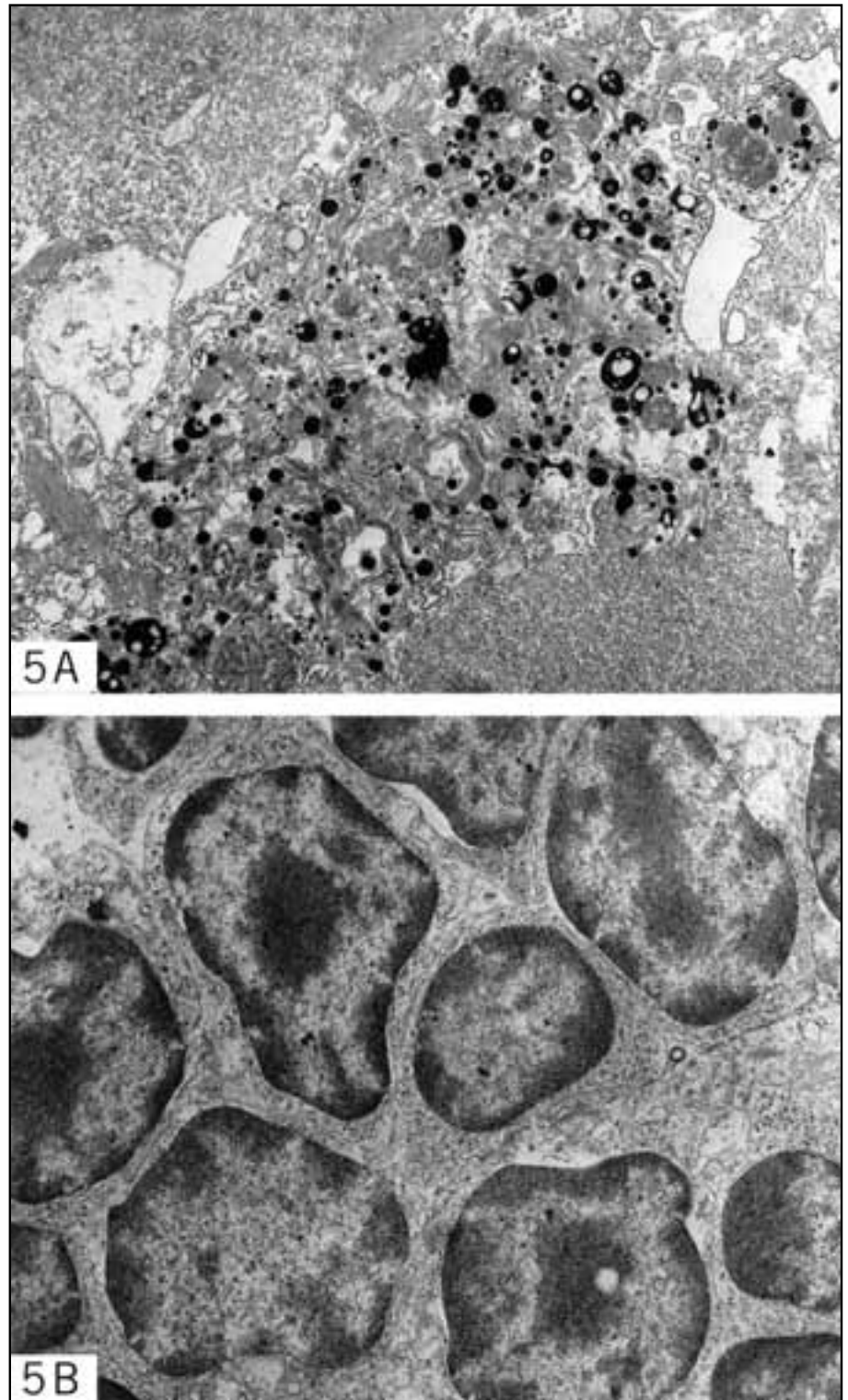
Ultrastructurally, the spleen of the old rats showed evidence of apoptosis of the lymphocytes, in which the nuclei of the cell was reduced in size with condensation of the nuclear chromatin. Some cells appeared to contain nuclear and cytoplasmic remnants enclosed by a cell membrane (*Figure 4A*). Evidence of cytoplasmic degenerative changes were apparent in other lymphocytes. In the melatonin treated animals, the incidence

of apoptosis was reduced. There was some evidence of vacuolar degeneration in the cytoplasm of the lymphocytes of these animals (*Figure 4B*).

In the thymus of old rats, the proliferated epithelial cells showed pronounced secretory activity with the appearance of numerous secretory granules at their apical margin (*Figure 5A*). Lymphocytic elements were scanty. In the melatonin-treated rats, typical lymphocytic cells formed the majority of the cortex. The cells appeared similar to those in the young controls (*Figure 5B*).

Figure 5.
Transmission electron
photomicrograph of thymus gland:
5A: Old rat showing numerous
secretory granules in the cytoplasm of
proliferated epithelial cells.
5B: Old rat treated with melatonin
showing more or less normal
lymphocytic cell population at the
thymic cortex. Uranyl acetate, lead
citrate (X5000).

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Discussion

The secretory product, melatonin, is released from the pineal gland of mammals especially during the dark phase of the light:dark cycle, and there are indications that it may influence some aging processes [26–30]. In the present study, exogenous melatonin in the drinking water was given to rats during a fixed circadian phase, i.e., during darkness when melatonin levels are normally elevated, to test whether it influenced the morphology and physiology of the thymus and spleen.

In the current study the concentration of melatonin in the drinking fluid was 25 µg/ml and each rat consumed approximately 12 ml per night. Thus, the calculated nightly intake of melatonin was on the order of 300 µg. Based on what is known concerning the gastrointestinal absorption of melatonin, this quantity likely caused blood levels to far exceed endogenously-generated melatonin concentrations that would normally be measured in the blood of young rats at night. Thus, these levels of melatonin, although normal in terms of their circadian phase (i.e., elevated at night), were

pharmacological in terms of their blood levels. However, given the wide compartmental variations in melatonin concentrations [31], how the amounts of melatonin consumed influenced melatonin levels in the rats generally is uncertain.

In the present study, the autoradiographic investigations revealed a significant reduction in the proliferative activity and the rate of DNA synthesis in spleen and thymus of old rats. These findings of a reduced lymphocyte proliferation are in agreement with other reports on the age-related decline of mitogenesis in lymphoid organs of rats [14, 15]. Regardless of the stimulus used, Pahlavani and Harris [18] found that both untreated and even melatonin-treated lymphocytes from old rats exhibited a significantly lower proliferation index than lymphocytes from young rats although melatonin treatment led to a significant higher proliferation rate of lymphocytes relative to that in non-melatonin treated cells. These findings are consistent with those observed in the present study.

Age-associated, free radical-related changes in the oxidation of essential macromolecules severely compromises the function of cells. For example, membranes damaged by free radicals become more rigid and exhibit decreased permeability as well as a reduction of their dynamic nature. Damaged DNA and proteins likewise compromise beneficial cellular processes. These changes lead to reduced function and may in fact result in death of the cells. One function of endogenously-produced melatonin may be to preserve optimal cell physiology by limiting oxidative damage to essential macromolecules [32]. Melatonin levels, however, diminish with age which may contribute to increased oxidative damage [33]. Thus, free radical damage that accumulates over a life time is generally considered consequential in aging and age-related diseases [20, 21]. It is possible that the loss of melatonin contributes to these processes [26, 33]. The deterioration of the immune system with aging, which leads to an increased morbidity and mortality from infections, appears to be related to decreases in specific lymphocyte functions and is clearly a function of increased age.

Histopathology of the spleens of the old, non-melatonin treated rats showed a severe loss of lymphoid elements with extramedullary hematopoiesis and increased number of megakaryocytes. Ultrastructurally, the spleen of the old rats showed evidence of apoptosis and cytoplasmic degeneration. In the thymus, there was a loss of cortical lymphocytes with apparent proliferation of epithelial cells, increased fibrovascular tissues, and occasional small nests or foci of lymphocytes. Ultrastructurally, in the thymus of old rats, the epithelial cells seemed to exhibit pronounced secretory activity and the lymphocytic elements were rare. These results are in agreement with Burek [34] who investigated the thymus and spleen of several strains of aged rats. Melatonin administration to the old rats reduced the incidence of apoptosis, megakaryocytic activity and hematopoiesis in the splenic lymphocytes. In the thymus, typical lymphatic cells form the majority of the cortical structure and fibrovascular tissues are rare.

Phelouzat et al. [35] reported that apoptotic deletion of activated mature lymphocytes is an essential physiological process implicated in both the regulation of the immune response and the control of the overall number of immunocompetent cells.

Administration of melatonin has been reported to increase mitogen-induced lymphocyte proliferation and IL-2 expression in mouse lymphocytes, two immune parameters that are consistently observed to change with age [2–4]. In addition, melatonin treatment stimulates natural killer cell activity, enhances antibody responses and increases antigen presentation by macrophages [19]. Melatonin has been reported to attenuate age-related thymic involution and partially restore cell-mediated delayed-type hypersensitivity responses. The immunostimulatory effects of melatonin are a particular interest in aging because, as already noted, a), melatonin levels decrease with increasing age and, b), there is an inverse relationship between blood levels of melatonin and involution of the immune system. Thus, the possibility that melatonin treatment might reverse the age-associated decline in immune function has generated significant interest.

Binding sites for melatonin have been identified in thymic cells as well as in peripheral blood lymphocytes and neutrophils. In the spleen as well, melatonin binding sites have been identified [36–39]. Maestroni and Conti [40] also have shown that melatonin, in addition to other possible mechanisms, acts directly on activated mouse spleen cells, increasing the release of opioid agonists, which enhance the immune response. Whether the beneficial effects of melatonin as seen in the present study are due to interaction with the specific melatonin receptors remains unknown.

In conclusion, the present work investigated the potential role of melatonin in maintaining the function and activity of splenic and thymic lymphocytes in old rats. The increased proliferative activity and the rate of DNA synthesis of old rats after melatonin treatment suggests that this indole may play a physiological role in the regulation of lymphoid function. Also, the data indicate that melatonin, besides other known functions, may participate directly in regulating immune physiology.

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