Role of the Pineal Gland in DMBA-Induced Hematotoxicity, Immunotoxicity and Mammary Carcinogenesis

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Abstract: There is substantial evidence that circadian disruption from modern life-styles, especially exposure to light at night, suppresses the pineal melatonin production and increases breast cancer risk. Environmental pollutants also may be involved in the etiology of breast cancer. This study addressed the effects of constant light (functional pinealectomy) alone or along with melatonin on DMBA-induced carcinogenesis. Female Sprague-Dawley rats (5-6 weeks-old) were randomly divided into five groups: (1) constant light (LL; n=20); (2) constant light plus melatonin (10mg / kg.b.wt; LL + Mel, n=20); (3) natural photoperiod (n=20); (4) vehicle (0.5% ethanolic phosphate-buffered saline, n=20); control (no treatment; n=10). All experimental manipulations started 15 days prior to DMBA and ended on the same day of DMBA treatment (single oral dose; 75mg /kg. b.wt). Animals were sacrificed at 5 months post-DMBA. Constant light exposure prior to DMBA produced anemia and lymphopenia (P<0.05). It also significantly increased the number of atypical lymphocytes (P<0.05). Melatonin did not reverse the effects of constant light and unexpectedly, it further reduced the hemoglobin concentration, suggesting severe anemia. The thymus in DMBA-treated rats showed signs of thymic involution; the spleen also displayed marked decrease in cellularity and had numerous apoptotic and necrotic cells. LL- exposure increased the state of immunodepression and caused further depletion in the thymocytes and splenic cells. Melatonin partially reversed the LL-induced lymphocytic depletion and caused fair improvement in the histological architecture of the lymphoid tissue. LL-exposure prior to DMBA seemed to stimulate the progression of existing mammary intraductal proliferations (IDPs) into ductal carcinoma in situ (DCIS). Melatonin only partially reversed those promoting effects of constant light. DMBA-treated rats exhibited extensive cytological alterations in the pineal gland. The relevance of the present work is that light exposure at night may function in a synergistic way with environmental pollutants to promote breast carcinogenesis.

Key words: Constant Light • Melatonin • DMBA • Mammary Carcinogenesis • Immune system • Ultrastructure • Pineal

INTRODUCTION

Cancer is a leading cause of death worldwide. However, evidence indicates that the majority of human cancers are potentially preventable because they can be attributed to external environmental factors such as dietary factors, cigarette smoking, radiation, occupational and environmental chemicals and socioeconomic status, in conjunction with endogenous factors [1]. Breast cancer is the most frequent cancer in women both in developed and the developing world; although breast cancer rates are higher in more industrialized nations, the incidence rates are continuously rising in developing countries [2]. In Egypt, breast cancer accounts for 35% of total cancer cases according to official statistics of the National Cancer Institute [3]. This has led to investigate etiologic factors that related to industrialization. In this context, exposure to environmental chemicals such as poly aromatic hydrocarbons (PAHs) has been shown to be involved in the etiology of breast cancer. PAHs are commonly found in cigarette smoke, industrial emissions, chimney soot, charbroiling and smoked meats [4].

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One member of this family, 7,12-dimethylbenz[a]anthracene (DMBA), is well known as both an initiator and promoter of breast cancer [5].

Moreover, in recent years a great deal of attention has been focused on the role of circadian disruption from aspects of modern life in the etiology of breast cancer. Specifically, there is substantial evidence of the existence of a relationship between exposure to light at night and breast cancer [6]. This relationship is supported by laboratory studies which have shown that light at night through the suppression of the pineal hormone melatonin, promotes carcinogenesis in human breast tissue [7]. Also, epidemiologic studies of shift workers have provided several lines of evidence that light at night may be a risk factor for breast cancer [8]. Furthermore, both the removal of the pineal gland and exposure to constant light (24h a day, LL) can independently cause an increase in mammary carcinogenesis in experimental animals, while administration of exogenous melatonin and light deprivation decrease mammary tumorigenesis [9].

The influence of constant light exposure on DMBA-induced mammary carcinogenesis has been studied in rats under different conditions of constant light regimen, but the results of these previous investigations were inconsistent [6]. Also, it has been observed that administration of melatonin to LL-exposed rats inhibits the development of mammary carcinogenesis [10]. In a previous study [11] administration of exogenous melatonin, for 15 consecutive days prior to DMBA-treatment, to virgin female rats kept in normal photoperiodic conditions inhibited DMBA-induced mammary carcinogenesis and protected the bone marrow from the damaging effects of DMBA. One of the mechanisms by which melatonin was thought to inhibit breast carcinogenesis is through increase the immune response [12].

It has been shown that neoplastic processes have been correlated with morphological and biochemical alterations in the pineal gland [13, 14]. Nevertheless, only limited number of studies previously investigated the effect of DMBA on the pineal ultrastructure and the results obtained were puzzling. For example, the first study of the effect of DMBA on the pineal ultrastructure was based on experimental manipulations known to enhance the pineal functions (Anosmia, underfeeding or cold exposure) and demonstrated that the pinealocytes of DMBA-treated rats have increased lipid content, suggesting an augmentation of the pineal metabolic activity [15]. On the other hand, Karasek et al. [16] investigated the pinealocyte ultrastructure in Fischer rats bearing an advanced (14th) passage DMBA-induced mammary carcinogenesis and observed no significant differences in ultrastructure of pinealocytes between control and tumor-bearing rats.

Therefore, the present study is a follow up of our early investigation and it aimed to assess the influence of the pineal gland as an oncstatic gland on DMBA-induced carcinogenesis in rats. In this view, the role of the pineal gland was evaluated through 2 experimental manipulations: 1) exposure of rats to constant light (Functional pinealectomy) in order to eliminate the circadian rhythm of melatonin and further depress day and night levels of the hormone; 2) melatonin administration to constant light-subjected rats in a trial to reverse the effects of constant light-exposure. To assess whether the induced mammary carcinogenesis involves changes in the pineal gland, we examined the ultrastructural changes in the pinealocytes of rats receiving DMBA, independent of constant light exposure whose effects on pinealocyte ultrastructure have been extensively studied [17, 18]. We also evaluated the effect of DMBA, constant light/DMBA and constant light plus melatonin/DMBA on some parameters of the immune system, including the structural architecture of lymphoid organs (Thymus and spleen) and some blood cell indices.

MATERIALS AND METHODS

Chemicals: DMBA (7,12-dimethylbenz[a]anthracene) and Melatonin (N-acetyl-5-methoxytryptamine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DMBA was dissolved in corn oil, to get a final concentration of 75mg/1ml of corn oil. Melatonin was prepared in ethanol and diluted to a final concentration of 10 mg melatonin/1ml of 0.5% ethanolic phosphate-buffered saline (PBS). The bottles of melatonin solution were covered with aluminum foil and kept in refrigerator and fresh solutions were prepared every two days.

Animals: Four to Five weeks old, virgin female Sprague-Dawely rats were obtained from the breeding colony of the Ministry of Health (Helwan-Egypt). The animals were weighed, group-housed (5 animals per cage), offered food and water ad libitum and kept in a well-ventilated animal facility under natural photoperiod conditions. All procedures were in accordance with institutional guidelines and follow the Guide for Care and Use of Laboratory Animals.
Experimental Design: After one week of acclimatization, virgin female rats (5-6 weeks-old) were randomly divided into five groups. The first group of animals (LL Group, n=20) was maintained under constant lighting conditions (LL, 300 Lux) for 15 consecutive days. Continuous light is known to suppress the pineal function (Functional pinealectomy) and abolish the day-night rhythm of melatonin secretion [19]. The light was provided by cool white fluorescent tubes. To ensure that each cage of animals received the same amount of light, cages were placed in a circle equidistant from the light source. Also, cages were rotated on each rack once per week to minimize the lack of homogeneity in light level. The second group of animals (LL + Mel Group; n=20) was subjected to the same experimental manipulation as the first group (LL Group) but they received a daily dose of melatonin (10mg / kg.b.wt) according to Lenoir et al. [20] by oral intubation in the late afternoon (4.00-6.00 pm). The 3rd and 4th groups were used as controls for the first and second groups. Unless otherwise specified, they received the same vehicle corresponding to the dose, route of administration and treatment time period. Briefly, control rats for the LL Group (n=20) did not receive any vehicle, but instead they were kept under natural photoperiod conditions (light-dark cycle LD). Control animals for LL+ Mel Group (n=20) were kept under constant lighting conditions (LL) and administered 0.5% ethanolic phosphate-buffered saline in the late afternoon. The fifth Group of rats (n=10) served as negative control animals, they did not undergo any experimental manipulations.

On the 16th day, rats from the 1st - 4th groups (now at 51-58 days of age) were given a single oral dose of 75mg /kg. b.wt of DMBA in 1ml of corn oil according to Lenoir et al. [20]. Based on previous studies, the susceptibility of the mammary gland to DMBA carcinogenesis has been shown to be strongly age-dependent and is maximal when DMBA is administered to animals between the ages of approximately 45-60 days [21]. Accordingly, the ages of the rats in the current study were thought to be suitable for induction of carcinogenesis. Because rats of the 3rd and 4th groups were not subjected to any experimental manipulation prior to the administration of DMBA, they designated as positive control for the incidence and histopathology of neoplasm. Animals of the fifth group were treated with a single oral dose of corn oil alone and served as negative control.

Rats were weighed weekly, palpated for mammary tumors detection once a week (starting two weeks post DMBA administration) and monitored daily for any signs of toxicity. The study had to be terminated at 150 days post-DMBA administration (Earlier than intended) because the mortality rate began to increase in the second group of treated animals (LL+Mel), to prevent any imbalance in the number of animals. All surviving animals including negative control group were sacrificed by decapitation under light ether anesthesia. Upon sacrifice, blood samples were collected in heparinized tubes for hematological analysis and lymphoid tissue, mammary glands as well as pineal glands were harvested for histopathological studies. Rats that died before the end of the experiment were excluded from the study.

Hematological Analysis: Total red blood cells (RBCs) count, hemoglobin concentration (gm/dl), total leukocyte (WBCs) count and differential leukocyte count were determined by standard techniques [22].

Histopathological Analysis

Light Microscopy: The thymus and spleen were excised and prepared for further processing. Also, both right and left cervical (#1), thoracic (#2 and #3), abdominal (#4) and inguinal (#5 and #6) mammary glands attached to the skin were removed from each rat. All tissues were processed for routine histopathological analysis using standard protocols. Criteria for diagnosis and classification of intraductal proliferations and other epithelial abnormalities in the mammary glands are mentioned elsewhere [11]. Briefly, Intraductal epithelial proliferations are classified into three groups: usual epithelial hyperplasia, atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS).

Because no palpable tumors were detected until the end of the study and only ductal carcinoma in situ (DCIS) was identified, incidence was calculated as the number of animals with lesions that meet the criteria for ductal carcinoma in situ (DCIS) compared to the total number of animals in the group.

The frequency of occurrence of various mammary lesions was assessed in the mammary glands; lesions that meet the criteria for usual ductal hyperplasia or atypical ductal hyperplasia were counted as intraductal proliferation (IDP) without categorization as previously described [11]. The frequency of IDPs and DCIS was calculated per gland (cervical, thoracic, abdominal and inguinal) per animal. Then, the values obtained for each of the glands were pooled and expressed as an average value for each animal. Finally, the mean and standard error were calculated for each group.
Electron Microscopy: The pineal glands were transferred to 3% (v/v) glutaraldehyde in 1% sodium cacodylate buffer (PH 7.4) at 2-4°C. After fixation, tissues were processed using standard protocols. Briefly, ultrathin sections were cut with a diamond knife, picked on copper grids, stained with saturated uranyl acetate and lead citrate and examined on the Transmission Electron microscope of the Military Medical Academy, Cairo, Egypt.

Statistical Analysis: Statistical analysis was performed using ANOVA (SPSS software, version 10.0; SPSS Inc. Chicago, IL, USA). Post-Hoc comparisons of pair-wise means were made using Least Significant Difference (LSD) test. The results were presented as mean ± standard error of the mean (SEM). Differences between group means were considered statistically significant if $P$-value > 0.05.

RESULTS

Rats of all treated groups did not show any significant changes in their body weight (Data not shown) when compared to control animals. Nevertheless, constant light/DMBA and constant light + melatonin/DMBA treated rats exhibited noticeable higher rates of mortality than DMBA alone-treated rats (Data not shown). The cause of deaths could not be established, but it can be attributed to severe anemia as indicated by morphological changes in erythrocytes and marked decreases in both erythrocyte counts and hemoglobin levels in the surviving animal of these groups, as discussed below.

Hematological Analysis: As shown in Table (1), by five months, the erythrocyte counts and hemoglobin levels of vehicle control (oil-treated) and DMBA-treated rats were not statistically different ($P > 0.05$). Exposure of rats to constant light alone or along with melatonin treatment for 15 consecutive days prior to DMBA administration (LL/DMBA, LL+Mel/DMBA groups, respectively) caused a significant decrease ($P < 0.05$) in both erythrocyte counts and hemoglobin levels. However, the hemoglobin level was apparently lower for LL+Mel/DMBA group than those for other groups.

In DMBA-treated rats the erythrocytes appeared normal with slight variance in size. (Fig. 1A). Constant light exposure (With/without melatonin administration) prior to DMBA-treatment induced marked changes in the morphology of erythrocytes, albeit these changes were more evident with melatonin administration. Specifically, peripheral blood smears from rats exposed to constant light alone or plus melatonin prior to DMBA exhibited relatively small erythrocytes (Microcytes) and ring shaped erythrocytes, compared to those from DMBA alone-treated rats (Figs. 1A-1D). Erythrocyte inclusions were also seen in some erythrocytes. These inclusions appeared as ellipsoid, violet-stained, finely structured rings in the erythrocytes (Fig. 1B). Moreover, the erythrocytes varied greatly in size (anisocytosis, Figs. 1B-1D) and shape (Poikilocytosis, Figs. 1C & 1D). Some microspherocytes appeared as strikingly small erythrocytes in which the central light areas were absent or only faintly visible (Fig. 1D).

DMBA administration induced a significant decrease in total leukocyte count ($P < 0.05$, Table 1) at five months post treatment. In contrast, total leukocyte numbers were significantly higher ($P < 0.05$) in LL/DMBA and LL+Mel/DMBA groups than in their corresponding DMBA-treated rats. Thus the values obtained for these two groups approached those of the controls.

In DMBA-treated rats lymphocyte and neutrophil percentages were significantly decreased ($P < 0.05$), whereas monocytes and esinophils percentages did not show any significant decreases ($P > 0.05$), as compared with their corresponding controls (Table 2). Leukocytes with ring-shaped nuclei and significant numbers of atypical lymphocytes were found in the peripheral blood of DMBA-treated rats. The nuclei of some atypical lymphocytes exhibited indentations with occasional bilobing (Fig. 1A). The percentage of lymphocytes was significantly decreased ($P < 0.05$) in rats subjected to constant light alone or along with melatonin administration, when compared to controls and DMBA-treated rats (Table 2), this decrease was accompanied with a significant increase in atypical lymphocytes ($P < 0.05$, Table 2, Figs. 1B & 1D).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>DMBA</th>
<th>LL/DMBA</th>
<th>LL+Mel/DMBA</th>
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<tr>
<td>RBCs ($x 10^6$)</td>
<td>6.1±0.27</td>
<td>6.05±0.66</td>
<td>2.73±0.49</td>
<td>2.6±0.05</td>
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<td>Hb (gm/dl)</td>
<td>14.7±0.7</td>
<td>14.33±0.37</td>
<td>12.46±0.85</td>
<td>10.62±0.38</td>
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<tr>
<td>WBCs ($x 10^9$)</td>
<td>4.5±0.28</td>
<td>2.93±0.58</td>
<td>4.33±0.46</td>
<td>4.33±0.14</td>
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</table>

Data represent means ± SEM. DMBA, 7,12-dimethylbenz(a)anthracene; LL/DMBA, constant light exposure prior to DMBA; LL + Mel/ DMBA, constant light exposure along with melatonin administration prior to DMBA. *= $P < 0.05$ vs control animals, ^= $P < 0.05$ vs DMBA-alone-treated rats.
Fig. 1: Stained blood films from female rats (5 months post-DMBA administration). (A) is from DMBA-treated rat showing fairly normal erythrocytes, and atypical lymphocyte with hyperchromatic bilobed nuclei and abundant basophilic cytoplasm. (B) and (C) are from constant light /DMBA-exposed rats, and (D) is from constant light plus melatonin/DMBA-treated rat. (B- D) are showing microcytes (m) ring-shaped erythrocytes (r), microsperocytes (ms), atypical lymphocytes (L), ring-like leukocyte with esinophilic granules (ri); note the presence of erythrocyte inclusions in the form of ellipsoid rings (arrow), and great variance in erythrocytes size (anisocytosis) and shape (polikilocytosis). (Giemsa stain, x 1000).

Table 2: Changes in differential leukocyte count (Mean percentage ± SEM) of virgin female rats exposed to constant light with/without melatonin for 15 consecutive days prior to DMBA administration.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>DMBA</th>
<th>LL / DMBA</th>
<th>LL+Mel/ DMBA</th>
</tr>
</thead>
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<tr>
<td>Lymphocytes</td>
<td>67.9 ± 0.34</td>
<td>52.7 ± 0.7</td>
<td>42.66 ± 1.34</td>
<td>44 ± 1</td>
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<tr>
<td>Atypical lymphocytes</td>
<td>0 ± 0</td>
<td>38.05 ± 0.59</td>
<td>49.34 ± 0.34</td>
<td>49.7 ± 0.7</td>
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<tr>
<td>Neutrophils</td>
<td>25.75 ± 0.43</td>
<td>6.48 ± 0.52</td>
<td>6.67 ± 0.67</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Monocytes</td>
<td>3.52 ± 0.19</td>
<td>1.85 ± 0.35</td>
<td>1.33 ± 0.13</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1.41 ± 0.25</td>
<td>0.93 ± 0.25</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Basophils</td>
<td>1.41 ± 0.25</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Data represent means ± SEM, determined after 5 months of DMBA administration. For abbreviations see Table (1). **P < 0.05** vs control animals, *P < 0.05* vs DMBA-alone-treated rats.

Unlike lymphocytes, the percentages of monocytes and neutrophils were not appreciably affected (P > 0.05, Table 2) by exposure to constant light prior to DMBA treatment, but their values were still significantly lower than those for the controls. Melatonin administration to constant light-subjected rats produced similar effects on monocytes, but it significantly reduced the percentage of neutrophils (P < 0.05, Table 2). In all rats exposed to constant light (LL/DMBA and LL+Mel/DMBA groups), there were numerous leukocytes with ring-shaped nuclei.

Eosinophils were not observed in the blood smears of constant light-exposed rats, irrespective of melatonin administration and this absence was statistically significant when compared with the percentages of eosinophil in the control and DMBA-treated rats. However, some ring-like leukocytes appeared with esinophilic granules in the
peripheral blood smears of rats that exposed to constant light or received melatonin along with constant light exposure (Fig. 1C). Basophils were not observed in any of the treated groups, but it still in the range of normal values.

**Histopathological Analysis**

**Light Microscopy**

**Lymphoid Tissues:** The thymus gland of DMBA treated rats showed obvious depletion of lymphocytes accompanied by increased necrosis, especially in the inner cortex. Also the lymphocytes appeared shrunken compared to those of control rats (Figs. 2A & 2B). Such reduction in the number and size of the cortical lymphocytes affect the cortico-medullary ratio which seemed to be decreased, causing loss of demarcation between cortex and medulla (Fig. 2C). The medulla had an extensive number of reticuloepithelial cells and Hassall’s corpuscles which were mostly cystic. It also had cystic dilatation of epithelial cells filled with homogenous esinophilic material (Fig. 2C). Furthermore, the interlobular septa became increasingly thickened and hyalinized

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**Fig. 2:** Histological sections of thymus. (A) from control rat showing normal structure of cortex with densely-packed cortical lymphocytes; an interlobular septum (s) is demonstrated. (B & C) from DMBA-treated rat revealing lymphocyte depletion accompanied by necrosis (arrows), thickened interlobular septum (s), epithelial cells (e), prominent blood vessel (bv); note the cystic dilatation of epithelial cells filled with esinophilic homogenous material (c). (D) from LL/DMBA rat revealing further marked lymphocyte depletion. (E & F) from LL+ Mel /DMBA rat showing apparent increase in the lymphocyte population with less necrosis; note the thickened interlobular septum (s) and the numerous calcified Hassall's corpuscles (h). (H & E x. 400).
Fig. 3: A-G high magnification of spleen sections. (A-C) from DMBA-treated rats, and (D-F) from LL/DMBA-treated rats; note the decreased cellularity of both white pulp and red pulp areas, the complete depletion of some areas leaving empty and vacuolated spaces (V), necrosis and apoptosis (thin arrows), thickened trabeculae (T), and hemosiderosis (h); note also that these alterations are more pronounced in spleen of LL/DMBA-treated rats (Panels D-F) which shows complete degeneration of splenic cords and hyalinization (thick arrow) in the red pulp (Panel F). (G & H) from LL+Mel/DMBA-treated rats demonstrating increased population of splenic cells, and decreased apoptosis/necrosis. (H & E x. 400).

white pulp areas showed lymphocyte depletion with the appearance of numerous shrunken, necrotic as well as apoptotic lymphocytes (Fig. 3A). Additionally, many white pulp areas appeared completely depleted from the lymphoid cells leaving empty and vacuolated spaces (Fig. 3B). The red pulp was intensely congested with red blood cells and showed lymphocytic hypopcellularity as well as increase in hemosiderin pigments (hemosiderosis); also the trabeculae appeared more thickened and hyalinized (Fig. 3C). Spleens from rats exposed to constant light prior to DMBA treatment (Figs. 3D-3F) were remarkably similar to those of DMBA-treated rats. However, they showed further hypopcellularity in the white pulp (Fig. 3D). Also, complete degeneration of splenic cords and hyalinization (disposition of esinophilic substances) were frequently

Constant-light exposure prior to DMBA administrations caused further depletion in the thymocytes which appeared shrunken and necrotic (Fig. 2D). Melatonin administration to constant light-exposed rats produced slight histological improvement in the thymus tissue after DMBA administration. The density of the cortical lymphocytes was apparently increased and the number of necrotic thymocytes seems to be decreased, but the thickened interlobular septa were still clearly visible (Fig. 2E). The medullary region also still had numerous Hassall’s corpuscles which were mostly cystic (Fig. 2F).
seen in the red pulp (Fig. 3F). The histological architecture of spleens from rats received constant light plus melatonin prior to DMBA showed fair histological improvement; in that, they showed increased population of splenic cells and decreased necrosis/apoptosis (Figs. 3G & 3H).

**Mammary Gland:** A single oral dose of DMBA (75mg/ k.b.wt) induced DCIS in 100% of rats (Fig. 4). In contrast, no palpable mass was detected in any animal examined. The frequency of occurrence DCIS and IDPs (hyperplasia and atypical hyperplasia) per rat was $77.29 \pm 1.11$ and $18.27 \pm 1.053$, respectively (Fig. 5). The histopathologic picture of DCIS was variable, but the major histologic variant was solid type.

As shown in Fig (4), all rats exposed to constant light for 15 consecutive days prior to DMBA-administration (LL/DMBA) developed DCIS (100% incidence), similar to LD-housed DMBA-treated animals. The frequency of occurrence DCIS, however, increased significantly in LL-exposed animals ($p < 0.05$; Fig. 5) compared with their LD-housed DMBA-treated counterparts; this increase was accompanied with a significant decrease in the frequency of IDPs occurrence ($p < 0.05$; Fig. 5).

Figure (4) shows that the incidence of DCIS in LL-housed animals was not affected by melatonin administration. That is, similar to DMBA-alone and LL/DMBA groups, 100% of animals subjected to constant light along with melatonin administration (LL+Mel group) for 15 consecutive days developed DCIS after DMBA treatment.

Moreover, although no significant difference was found in the frequency of DCIS occurrence between LL+Mel / DMBA female rats and DMBA-alone treated animals ($p > 0.05$, Fig. 5), there was a trend toward decreasing the frequency of DCIS occurrence in LL+Mel / DMBA females compared to LL / DMBA animals ($p > 0.05$, Fig. 5). In contrast, the frequency of occurrence of mammary intraductal proliferations was significantly decreased in LL+Mel / DMBA group ($p < 0.05$, Fig. 5) compared to DMBA and LL / DMBA groups.

Histopathological investigation of mammary glands from rats exposed to constant light prior to DMBA-administration showed that one hundred percent of animals had DCIS of solid pattern in all regions of mammary glands examined. Central necrosis and mitotic figures were noticed in the expanded ducts with neoplastic cells (Figs. 6A & 6B). Atypical ductal hyperplasia and hyperplasia of usual type was also observed in all animals. Periductal stromal fibrosis (desmoplasia) was also evident (Fig. 6A).

Exposure to constant light plus melatonin treatment prior to DMBA produced DCIS of both solid and comedo patterns in all regions of mammary glands, along with usual ductal hyperplasia and atypical ductal hyperplasia. However, the solid pattern of DCIS was more frequent (Figs. 6C & 6D), in which the distended ductal lumen was completely obliterated by sheets of neoplastic cells of greater homogeneity with nuclear hyperchromasia, variable nuclear grade and mitosis (Figs.6C & 6D). Slight periductal fibrosis and stromal reaction were noticed as well (Fig. 6D).

**Electron Microscopy:** The ultrastructure of the pineal gland of control rats was similar to that of normal adult albino rat previously described in detail by many authors [23, 24]. Therefore, the present study mainly describes the
Fig. 6: A & B sections of mammary glands from female rats exposed to constant light for 15 consecutive days prior to DMBA (5 months post-DMBA treatment), showing ductal carcinoma in situ (DCIS) of solid type, and periductal stromal fibrosis (arrows); note the presence of numerous mitotic figures. C & D sections of mammary glands from female rats exposed to constant light plus melatonin prior to DMBA-administration, revealing DCIS of solid type; note the presence of nuclear hyperchromasia and mitosis (arrows) in Panel C, and periductal fibrosis in Panel D. (H&E., 400).

Effects of DMBA administration on the ultrastructural morphology of the pineal gland. As shown in Figs (7-12), a single oral dose of DMBA (75 mg/kg. b. wt.) produced marked changes in the fine structure of all cell types of the pineal parenchyma (light pinealocytes, dark pinealocytes and interstitial cells). The cytoplasm of all cells appeared extensively vacuolated (Fig. 7) with aberrantly shaped cellular organelles. However, none of the various ultrastructural changes were more dramatic than the occurrence of destructive alterations in the mitochondria. In this regard, bizarre mitochondria of varying shape, size and internal organization were found in the pineal cells of all treated animals (Figs. 7-12). Numerous mitochondria appeared swollen with pale matrix and were almost devoid of cristae (Figs. 8-12). Others appeared as empty vacuoles due to the complete destruction of cristae (e.g. Figs. 9 & 10). However, various mitochondria containing intra-mitochondrial dense bodies (Fig. 9) were observed in the cytoplasm of many cells. Also, deformed mitochondria with abnormally positioned (longitudinal) cristae were seen (Fig. 8). Besides, many other mitochondria appeared with very dense matrix and barely identified high electron-dense cristae (Fig. 10). Such various mitochondrial profiles were often found in the same cell.

In some pineal cells, much of the smooth and rough endoplasmic reticulum was lost and many free ribosomes were observed in the cytoplasm (Figs. 8-10). In other cells, however, there were scattered and disorganized profiles of rough endoplasmic reticulum, which were no longer found in parallel arrays, but became greatly swollen and fragmented (Fig. 11). Golgi complexes were not usually seen in cells that mostly lost rough endoplasmic reticulum, but they were often observed in those cells that contain dilated and swollen profiles of rough endoplasmic reticulum. However, the morphology and size of the Golgi complex showed obvious changes (Fig. 11).

A great number of lysosomal derivatives were observed. Usually, these derivatives were seen as membrane-lined segregations of cytoplasmic components. Some of these cytoplasmic sequestrations were formed of dense particles in a fairly homogenous matrix and were...
Fig. 7: Low power micrograph of pineal parenchyma from DMBA-treated rat, demonstrating numerous cytoplasmic vacuoles (v), fragmentation of nucleoli with loss of the nucleonemmas (nu), discontinuous nuclear membrane (black arrow) and nuclear fragmentation (karyorrhexis; white arrow). c, cytoplasm; n, nucleus. x 3000.

Fig. 8: Electron micrograph of a pinealocyte from DMBA-treated rat showing various forms of bizarre-shaped mitochondria (m). Many mitochondria are swollen and lacking most of cristae; others appear deformed with abnormally-positioned cristae. Note the presence of numerous free ribosomes (r) and the scarcity of smooth and rough endoplasmic reticulum (rer) which is dilated, disorganized and attached with only few ribosomes. Note also the presence of focally widened perinuclear space (ns), cytoplasmic vacuoles (v), cytoplasmic sequestrations (s). x 10000.

Fig. 9: Electron micrograph of a pinealocyte from DMBA-treated rat. The cytoplasmic fine structure closely approximates that illustrated in the previous picture, but more abundant free ribosomes (r) and more forms of bizarre-shaped mitochondria (m) are seen. Mitochondria appeared as empty vacuoles, or contained intramitochondrial dense cores. Note the cytoplasmic vacuoles (v), the discontinuity of the nuclear membrane (black arrow) and the nuclear fragmentation (white arrow). x 10000.

Fig. 10: Electron micrograph of a pinealocyte from DMBA-treated rat, displaying ovoid and longitudinal mitochondria (m) with extensively compact matrix and hardly-defined cristae, along with abnormally shaped mitochondria. There is a scanty disorganized rough endoplasmic reticulum, but there are numerous evenly-distributed free ribosomes (r). x 10000.

Fig. 11: Electron micrograph of a pinealocyte from DMBA-treated rat. In contrast to the paucity of endoplasmic reticulum shown in Figs. 8-10, the pinealocyte in this micrograph has numerous profiles of disorganized rough endoplasmic reticulum (rer) which appear swollen and fragmented, with loss of parallel arrays. Note the presence of swollen Golgi complex (g), numerous free ribosomes (r), mitochondria (m), phagosomes and residual bodies (arrows) and lipid droplets (lp). x 15000.

Fig. 12: Electron micrograph of a pinealocyte from DMBA-treated rat. The cytoplasm contain large bizarre-shaped mitochondria, large cavities of rough endoplasmic reticulum (rer) associated with only few ribosomes and lipid droplets (lp). x 15000.

occasionally continuous with the cisternae of the endoplasmic reticulum (Fig. 8). Various other cytoplasmic sequestrations, consisting of membrane-bound areas of homogenous cytoplasm were also seen (Fig. 8). Also, phagosomes and residual bodies of various size and composition were observed (Fig. 11). Moreover, numerous cytoplasmic vacuoles (Figs. 7-12) as well as lipid droplets (Fig. 11 and 12) were present throughout the cytoplasm.

The nuclei and nucleoli showed great variability in size, structure and shape (Fig. 7). Some cells appeared with discontinuous nuclear membrane and showed destructive fragmentation of the nucleus (karyorrhexis), so, parts of the nuclear chromatin were distributed irregularly throughout the cytoplasm (Figs. 7 and 9). Also, a well-marked focal widening of the perinuclear space was seen in some pineal cells (Fig. 8). The nucleoli appeared fragmented into diffuse structures, with loss of the nucleolnema (Fig. 7).

DISCUSSION

In a previous study, preventive treatment with melatonin (10 mg/k. b.wt.) for 15 consecutive days prior to DMBA-administration produced marked inhibition of mammary carcinogenesis and appeared to protect the bone marrow from damaging effects of DMBA [11]. The current study extended our initial investigation and determined the effects of eliminating endogenous melatonin signal, through constant light exposure (functional pinealectomy), on DMBA-induced hematotoxicity, immunotoxicity and mammary gland carcinogenesis. It also elucidated the effect of exogenous melatonin on these parameters in functionally pinealectomyzed rats. Finally, it demonstrated the effects that DMBA might have on the pineal gland itself. Constant light exposure seemed to stimulate the toxic effects of DMBA. Melatonin administration to constant light-exposed rats partially reversed the effects of constant light and in case of erythrocyte indices, it appeared to increase the severity of anemia. DMBA also produced extensive cytological alterations in the pineal gland, suggesting a role of the pineal gland in mammary carcinogenesis.

Blood cell indices (RBC, WBC, DLC counts) are good indicators of systemic response to external stimuli and any changes can be therefore reflected in their morphology and distribution in the blood. In the present study, a single oral dose of DMBA (75mg/kg b.wt.) did not appreciably affect the hemoglobin concentration or the erythrocyte count, as determined after 5 months post-DMBA administration. As discussed elsewhere [11], these results agree with those of N’jai et al. [25] who reported non-significant alterations in red blood cell numbers, hematocrit or platelet numbers of rats after intraperitoneal (IP) or oral DMBA treatment. The same authors reported that DMBA selectively targets the precursors of leukocytes in the bone marrow rather than those of red cell or thrombocyte.

On the other hand, exposure to constant light for 15 consecutive days prior to DMBA administration induced significant reduction in hemoglobin concentrations and erythrocyte counts, indicating an anemia. Also, contrary to expected results, melatonin administration to constant light-exposed rats produced further decrease in the hemoglobin concentrations and erythrocyte counts, suggesting severe anemia. The occurrence of anemia in these animals was confirmed by erythrocyte morphology. Specifically, the observation of microcytes and ring-shaped erythrocytes indicates the presence of advanced hypochromic anemia [26]. Also disturbances in erythrocyte morphology (anisocytosis and poikilocytosis) may indicate ineffective erythropoiesis, inflammation, kidney disorders [27], or secondary anemia, usually in cases of tumor; the ellipsoid rings noted in erythrocytes in the current study are called “cabot rings” and probably consist of fibers from the mitotic spindles; they are occasionally observed in all severe anemias, with no specific explanation [26].

The reduction in RBCs count and hemoglobin concentration may occur due to iron deficiency, or due to hemolytic or myelopathic conditions [28]. Therefore, constant light exposure prior to DMBA may induce one or more of these conditions causing the observed anemia. Also, given that the suppression of melatonin synthesis favors the mounting of an inflammatory response [29], constant light exposure prior to DMBA-treatment may result in the development of chronic inflammatory state; accordingly anemia of chronic disease (ACD) develop as a type of anemia, mimics iron deficiency, although there is no deficiency of iron per se; the iron instead is sequestered by mononuclear phagocytes and its availability for erythropoiesis is decreased [30].

The reasons why melatonin administration to constant light-subjected rats did not restore the levels of hemoglobin concentration or erythrocyte count are obscure. Considering the available literature, melatonin is known to have beneficial effects in many blood disorders including anemia [31, 32]. So, the discrepancies between the results of previous studies and those reported here could be due to the experimental protocols used in this
study which were based on pretreatment procedures prior to DMBA-administration, so as to discontinue treatments at the day of receiving DMBA. This suggestion is supported by the studies of Labonia et al. [31] in which treatment with 6 mg melatonin at night for 30 days in patients with anemia of chronic disease (ACD) caused significantly improved iron status and hemoglobin values, but these effects have been almost completely reversed within 2 weeks of discontinuing melatonin treatment. Nevertheless, these observations do not clarify the reasons for the occurrence of more severe anemia in melatonin-treated rats in the present work. This unexpected effect of melatonin could be related to removing of endogenous melatonin signal by constant light exposure. There are some reports demonstrating that eliminating the endogenous melatonin signal changes the exogenous melatonin’s dose response characteristics [33]. Such observation was also reported by Aubert et al. [34] who revealed that melatonin treatment of rats with DMBA-induced mammary carcinogenesis is not effective when applied to animals kept in constant light and, consequently, with suppression of the endogenous melatonin rhythm.

The current data revealed that DMBA induced an apparent decrease in the total leukocyte count, as well as lymphocyte, monocyte and granulocyte percentages. These effects of DMBA on the total and differential leukocyte counts are discussed elsewhere [11]. Concisely, hematopoiesis begins with hematopoietic stem cells (HSCs) that give rise to all blood cell lineages and is regulated by a tight network of cytokines and hormones, which are essential for the survival and differentiation of progenitor cells; this process can be altered by DMBA [25]. In addition, the observed lymphopenia may reflect adverse effects of DMBA on other lymphoid organs [35].

In the current work, exposure of female rats to constant light for 15 consecutive days (functional pinealectomy) before DMBA treatment (LL / DMBA) resulted in significant increases in the total leukocyte counts as well as atypical lymphocyte percentages and significant decreases in lymphocyte percentages, as compared to DMBA-alone treated rats. The current elevation in the total number of leukocytes may be related to the increase in the number of atypical lymphocytes. This suggestion is supported by the observation of lower total number of leukocytes under constant light conditions in rats [36]. It has been established that pinealectomy or any other procedure that inhibits melatonin synthesis and secretion produces a state of immunodepression that counteracted by melatonin treatment [37]. This was not the case in the present study in which melatonin administration to constant light-exposed animals (LL+Mel/DMBA group) did not restore the number of leukocytes. So, these results indicate that melatonin treatment did not reverse the lowering effects of constant light on white blood cells. As discussed earlier, this could be due to the removal of endogenous signal of melatonin under constant light exposure. Such suggestion is confirmed by the previously observed leukocyte enhancing effects of exogenous melatonin given to LD-housed rats prior to DMBA in an identical protocol [11].

In this study, rats treated with DMBA showed an increased number of atypical lymphocytes and ring-shaped leukocytes. Constant light exposure (with or without melatonin administration) prior to DMBA induced further marked increase in the number of atypical lymphocytes and ring-shaped leukocytes. According to Simon [38], atypical lymphocytes are nonmalignant actively proliferating leukocytes seen in the peripheral blood and produced in a variety of disorders. Also, ring shaped cells are determined in peripheral blood during myeloproliferative disorders [39]. The existence of these cells in the peripheral blood of treated animals in this study indicates an inhibition in the proliferation and differentiation of hematopoietic stem cells.

As regard to the immune system, the present results showed that in addition to the observed leucopenia, DMBA induced profound decrease in the cellularity of thymus and spleen. Constant light exposure prior to DMBA enhanced the degenerative effects of DMBA on the lymphoid tissues. Melatonin administration along with constant light did not reverse the profound lymphodepletion noted after DMBA treatment; although little improvement was observed in the histological architecture of the thymus and spleen of LL+Mel/DMBA rats. DMBA is well known to elicit immunosuppression by reducing cellularity in the thymus [40, 41] and spleen [35, 41], therefore, DMBA suppresses both humoral immunity and cell-mediated immunity [35]. Additionally, it is well established that the pineal gland through its principal hormone melatonin affects lymphoid tissue size, structure and function. Earlier studies have shown that exposure of rats to constant light (functional pinealectomy) caused involution of the thymus and increase in the number of pyknotic lymphocyte nuclei [42]. The present data strengthen those previous observations; specifically, the thymus of treated rats from all groups showed signs of involution which are similar to those induced by acute stress or steroid hormones administration. Previous observations on the DMBA-induced thymic involution suggested that thymic alterations are due not only to the...
increased cortisol level but also to a direct effect of DMBA on the thymic tissue, probably through the inhibition of DNA synthesis [40]. Since melatonin secretion under normal photoperiod conditions may antagonize the effect of steroids [42], the inhibition of melatonin synthesis and release by constant light exposure in this study may induce steroid-initiated destructive effect on the thymus, thus enhancing the effects of DMBA. Similarly, in this study, the structural alterations of spleen could be related to direct effects of DMBA or to inhibited melatonin secretion (caused by continuous light exposure) on the splenic tissue. This suggestion is supported by previous reports [35, 43]. It is not exactly known why suppressed immune functions were not significantly restored in constant light-exposed rats after the administration of melatonin. Again, this could be related to eliminating the endogenous melatonin signal by constant light exposure.

In the present study, oral administration of a single dose of DMBA (75mg/Kg.b.wt) produced ductal carcinoma in situ, as well as intraductal hyperplasia (intraductal proliferations, IDPs) in all mammary glands of treated rats. The significance of occurrence of intraductal hyperplasia in the mammary gland is that they are considered to be the precursors of carcinomas both in rodents and humans [44]. Exposure of rats to constant light (LL) for 15 consecutive days before DMBA-administration induced DCIS in 100% of rats and increased the frequency of DCIS occurrence as compared to their LD-housed DMBA-treated counterparts; however, constant light exposure inhibited the frequency of IDPs occurrence suggesting stimulation in the progression of existing IDPs into DCIS. The present data are consistent with previous investigations which showed a promoting effect of constant light-exposure on mammary carcinogenesis induced by DMBA [6, 9]. However, previous experiments on the effects of constant light on DMBA-induced mammary carcinogenesis in rats yielded conflicting results in which mammary tumors were either stimulated [10] or reduced [45]. These differences seem to be due to variations in the age of the rats at the time of first exposure to constant light which resulted in differences in mammary tissue development; this in turn could change tumor susceptibility [21, 46].

Several mechanisms have been suggested to explain the relationship between pineal gland and breast cancer. It has been hypothesized that loss of pineal function and the resulting decreased melatonin levels may increase reproductive hormones and, in particular estradiol levels, thus increasing the growth and proliferation of hormone-sensitive cells in the breast [9]. However, it has been recently shown that melatonin is directly oncostatic [12].

Administration of melatonin to LL-housed rats for 15 days prior to DMBA administration induced DCIS in 100% of rats, but produced a significant decrease in the frequency of occurrence of mammary DCIS and IDPs, indicating that exogenous melatonin only partially reversed the effects of functional pinealectomy. This finding is in agreement with those reported by Cos and Sánchez-Barceló [47] who observed that the tumor occurrence was reduced from 87% in pinealectomized rats to 63% in pinealectomized plus melatonin rats, whereas the tumor occurrence in melatonin-treated intact rats was still lower (27%). There is substantial evidence that the effects of melatonin administration are different depending on whether pineal gland is present or not [10, 48]. The presence of a daily rhythm of melatonin may affect the sensitivity of specific target tissue to this hormone. For example, administration of melatonin prior to the nocturnal increase in endogenous melatonin may be most effective because tissues are most sensitive to the hormone at this time. Pinealectomy abolishes the melatonin rhythm in rat, causing a loss in organ sensitivity to melatonin and may abolish the time-of-day sensitivity to exogenous melatonin. Therefore, Melatonin treatment is not effective when applied to rats in constant light and, consequently, with suppression of the endogenous melatonin rhythm [34]. Thus, the results reported here in which the incidence of DCIS in LL-housed animals was not affected by melatonin administration extend and confirm those earlier observations. Conclusively, the current data support the hypothesis that the pineal gland itself may influence the sensitivity of target organs to exogenous melatonin and that its presence is essential to the antitumoral and immunoenhancing effects of the hormone [10, 48]. This hypothesis is further confirmed by our previous study in which inhibitory effect of exogenous melatonin on DMBA-induced mammary carcinogenesis has been observed in female rats under similar experimental conditions [11].

Noticeable ultrastructural alterations were produced by DMBA in the pineal gland. The most extensive change was the presence of bizarre mitochondrial profiles of various shapes, sizes and internal organization. To our knowledge, no information is available on the mitochondrial alterations in the pineal cells of DMBA-treated rats. However, bizarre mitochondria have been observed in the pinealocytes after various other
In this study, the pineal cells of DMBA-treated rats showed extensively vacuolated cytoplasm with a great number of lysosomal derivatives including residual bodies, dense bodies, phagosomes and cytoplasmic sequestrations. The occurrence of similar lysosomal derivatives has been demonstrated in DMBA-treated rat tracheal explants [49]. It has been hypothesized that lysosomes play an important role in the carcinogenesis of polycyclic aromatic hydrocarbons. According to Allison et al. [56], these carcinogenic hydrocarbons are concentrated in the lysosomes and produce permeability changes in the lysosomal membranes, allowing the release of lysosomal enzymes which produce secondary effects. Nevertheless, Cos et al. [15] postulated that the increase in degenerative changes such as cyst formation in the pineal cells was not a consequence of direct damage induced by DMBA at pineal level.

Given that vacuolation of the pineal cells increases with increasing the physiological activity of the gland [57], the current data strengthen Relkin’s hypothesis, on the role of pineal gland in cancer, that at first the gland become hyperactive in an effort to secrete oncostatic substances, including melatonin in order to suppress the malignant growth, causing exhaustion and degeneration of the gland, when the carcinoma is well advanced [13]. Parallel findings of the exhaustion of the pineal gland and depression of melatonin rhythm with advanced stage of carcinoma have been reported by Bartsch et al. [14]. This may led to the assumption that the activity of the pineal gland may be increased in DMBA-treated rats in the current study in an attempt to secrete more melatonin which is the main hormone of the pineal gland to control the resulting DCIS. Nevertheless, it seems that DMBA has direct degenerative effects on the gland itself as appeared from the ultrastructural alterations. Since the pineal gland is partially outside the blood brain barrier, it is not surprising that DMBA may have direct influence on the pineal.

Altogether, the present ultrastructural investigation of the pineal cells of female rats after DMBA-administration strongly indicates that the activities of the pineal cells are affected by DMBA. However, beside exposure to DMBA itself, the process of carcinogenesis as well as the state of having DCIS may produce several changes in the ultrastructure of the pineal gland.

The relevance of the present work is that humans are exposed to light at night which has become essential part of modern life style. In particular women who work in rotating night shifts seem to have an increased risk of breast cancer after extended periods of rotating night work [8]. Also, exposure to DMBA through environmental
and dietary sources has been implicated in the development of breast cancer in humans. In this regard, postmenopausal women who consumed the most grilled or barbecued and smoked red meat over their life time, more than once a week, had a 47 percent greater risk of breast cancer compared with women who consumed such meats once a week or less [58].

In conclusion, constant light increases the susceptibility to DMBA-induced mammary carcinogenesis, increases the DMBA-induced immunodepression and induces anemia in female rats, melatonin was not able to completely reverse the effects of constant light. Thus light exposure at night may function in a synergistic way with environmental pollutants (such as DMBA) to promote breast carcinogenesis.

REFERENCES


