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Melatonin Functioning Through DNA Methylation to Constricts Breast Cancer Growth Accelerated by Blue LED Light-At-Night in 4T1 Tumor-Bearing Mice

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Abstract

In an attempt to reduce Carbon Dioxide production, lighting technologies are aiming toward the developing of energetically efficient illumination sources as light-emitting diodes (LEDs). LED lamps emit light at short-wavelengths close to the sensitivity peak of melatonin suppression and expected to further exacerbate circadian disruption and cancer risk from increasing exposure to artificial light-at-night (ALAN). We report the effect of ALAN (1×30 min per night) emitted from yellow incandescent or blue-white LED bulbs and melatonin treatment on urinary 6-sulfatoxymelatonin (6-SMT), tumor growth (volume and mass), and global DNA methylation in 4T1 inoculated Balb/c female mice. Blue ALAN significantly decreased 6-SMT, increased tumor growths, and promoted metastasis formation verses yellow exposure. In blue-treated mice, DNA methylation levels were decreased in tumor and liver cells but not in lung and spleen cells compared with yellow-treated mice. Melatonin treatment inhibited tumor growth, reduced metastasis formation, and equally induced hypermethylation under the two spectral compositions. 6-SMTshowed strong inverse and direct correlations with tumor volume and methylation level, respectively. Finally, melatonin treatment increased relative spleen mass with no spectral differences compared with controls.

We demonstrate for the first time that LED lighting can result in aggressive and rapid tumor growth compared with the counterpart incandescent technology, and our results strongly suggest that the mechanism of action by which this occurs is altering DNA methylation levels by ALAN-induced melatonin suppression. These findings further support evidence for the possible association between ALAN and hormone dependent cancer incidences and subsequently advance the need for developing sustainable lighting solutions which are more efficient but less harmful.

Keywords: Breast cancer biomarkers; Cosinor analysis; Epigenetic modifications; Light pollution; Tumor-free interval

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Abbreviations: ALAN: Artificial Light-At-Night; GDM: Global DNA Methylation; LED: Light Emitting Diode; RM2W-ANOVA: Two-Way Mixed Repeated-Measures ANOVA; RM3W-ANOVA: Three-Way Mixed Repeated-Measures ANOVA; 1W-ANOVA: One-Way

ANOVA; 6-SMT: 6-sulfatoxymelatonin

Introduction

The clinical effect of artificial light-at-night (ALAN) is a complex and provocative contemporary phenomenon. ALAN is increasingly recognized as a risk factor for human health, particularly in cancer progresses [1,2]. Evidence from epidemiological studies on variant types of circadian disruption especially, by shift work have indicated a disquieting association between cancer incidences and circadian disruption by ALAN. Recently, an extensive meta-analysis of 28 studies including shift work, sleep deprivation, flight attendances, and ALAN, demonstrated a robust association between circadian disruption and breast cancer risk [3]. Moreover, in the meta-analysis study a dose-dependent relationship was revealed between shift work and cancer risk with 16% increase in cancer risk at 10 years consecutive shift work. Several earlier epidemiological studies also provide clear support for the positive dose-dependent pattern between ALAN and cancer risk, particularly at high levels of exposure and increasing period of shift work [4,5]. Furthermore, epidemiological studies in visually challenged women and men provide sufficient evidence that cancer risk is inversely related to the degree of visual impairment [6,7]. In industrialized countries including Israel, breast cancer risk co-distributes with regions of high light pollution levels [8,9]. Additionally, ALAN has been demonstrated to enhance human breast cancer growth in nude rat xenografted tumor model [10]. ALAN exposure from fluorescent illumination (450 lux, 469nm) increased breast cancer growth in Balb/c female mice [11]. Similar results of accelerated carcinogenesis and mortality rates were also observed in rats even with ALAN of very low intensity [12].

The adverse cariogenic effects of light pollution are postulated to be mediated by the circulating levels of the pineal gland indole-hormone melatonin (N-acetyl-5-methoxytrptamine). The hormone synthesis and release from the gland are light sensitive and subjected to prompt suppression by light [13]. Therefore, under natural photoperiod condition the circulating levels of melatonin exhibits conserved daily rhythms with higher levels during the night compared with minimal levels during the day [14]. The spectral suppression of melatonin production displays a dose-dependent response with higher suppression at short-wavelengths [15]. Blue light emitted from energy-efficient light-emitting diodes (LEDs) is more powerful in suppression melatonin production than any counterpart lightning technology such as incandescent and fluorescent lightning [16]. Circadian disruption by ALAN is expected to increase cancer risk by lessening the typical nocturnal increase in melatonin production and secretion [1].

The anti-carcinogenic property of melatonin has been established by several epidemiological and experimental studies in variant tumors including breast and prostate cancers [17]. High risk for breast cancer susceptibility is recurrently reported in women exposed to excessive and continuous ALAN (i.e., night work, sleep deprivation) during the period when melatonin levels are boosted to maximum [18,19]. Melatonin has been demonstrated to decrease the development of prostate and breast cancers and to suppress metastasis development in mice exposed to ALAN [11]. In general, melatonin manifests its anti-carcinogenic activity by blocking cancer cell proliferations, stimulating anti-tumor immunity, and accelerating apoptosis in cancer cell lines [20]. The molecular mechanism of action linking between the ALAN-induced melatonin suppression and carcinogenesis is not fully characterized, but epigenetic modification of oncogenes is a promising candidate.

Epigenetics refers to heritable modifications in gene expression without changing the DNA nucleic acid sequences. These modifications are potentially reversible and regulated by a wide variety of cellular and environmental stimuli. Generally, epigenetic mechanisms include DNA modifications, histone remodeling, and non-coding RNA silencing [21,22]. Genomic DNA methylation is the most common and enduring epigenetic modification in which the 5-carbon position of the cytosine ring within CpG dinucleotides is methylated to 5-methylcytosin (5-mC) by methyltransferases [23,24]. Cancer cells present different types of epigenetic modifications including global hypomethylation and promoter hypermethylation of tumor suppressor genes [25]. Global hypomethylation can contribute to breast cancer development by promoting genomic instability, aberrant

activation of oncogenes, and stimulating expression of metastatic genes [26]. Promoter hypermethylation of breast cancer oncogenes can also induce cancer development by silencing of both tumor suppressor genes and proliferation regulatory genes, promoting cell proliferation [26]. A recent review has suggested a potential role of the pineal melatonin in mediating the carcinogenic activity of ALAN [27]. According to the reviewed data, the reduced circulating levels of melatonin trigger intracellular reactions that advance aberrant DNA methylations and stimulate oncogene expressions.

To date, research regarding light pollution has predominantly focused on ecology, behavior, and physiology, but not on characterizing the spectral composition of ALAN on cancer etiology. Exposures to LED lighting are expected to further increase the light pollution problem as higher irradiance and shorter-wavelength are emitted compared with counterpart traditional lightning technologies [28]. With the increase administration of LED lighting in our surrounding including TV, computer, and smart-phone screens, the need for evaluating the spectral threshold of ALAN for eliciting carcinogenic activity becomes increasingly significant mainly for prevention but also presumably for therapeutic purposes. Therefore, the present study was applied to characterize the spectral sensitivity to ALAN in breast cancer inoculated mice exposed to either incandescent or LED illuminations. Additionally, the study aimed to elucidate the underling mechanism by which the ALAN-induced carcinogenic activity is mediated in regards to melatonin suppression and global DNA methylation. We hypothesize that "if exposure to LED is the strongest risk factor in respect to melatonin suppression then promotion of tumor cell proliferation is expected in which aberrant DNA methylation plays a significant mediator role".

Materials and methods

Animals

Female Balb/c mice (4-5 wks, 20 ± 1 g) were purchased from Harlan Laboratories Ltd., (Jerusalem, Israel). The mice were maintained at the Technion Israel Institute of Technology Preclinical Research Authority under specific pathogen-free conditions. All experiments exploiting mice were approved by the institutional Animal Care and Use Committee of the Technion- Israel institute of Technology, Haifa, Israel (Protocol number: IL-019-01_2011) and all experiment procedures were conducted with approval from the Ethics and Animal Care Committee of the University of Haifa. All efforts were made to minimize the number of animals used and their suffering and no other methods besides using animals were available. Mice (5/cage) were housed in the animal facility in a climate-controlled room (22 ± 1 °C, 53 ± 7% relative humidity) in individually ventilated cages (37X19X13 cm) with 75 changes of air per hour (Tecniplast, SmartFlow; Buguggiate, Italy), given *ad libitum* access to total pathogen-free food (Altromin 1324; Lage, Germany; 19% crude protein, 4% crude fat, 6% Cellulose, 13% moisture, 7.5% ash, 11.9 MJ/Kg metabolizable energy), and sterilized tap water. At arrival mice were acclimated for three weeks prior to tumor cell inoculation, to short photoperiod (SD; 8L:16D Light/Dark cycle, lights on from 08:00 to 16:00h) of either incandescent ($\lambda_{Dominant}$=580 nm) lighting (60 W; OSRAM; Molesheim, France) or natural ($\lambda_{Dominant}$=460 nm) LED light (9 W; LEEDARSON LIGHTING; Xiamen, China) at 350 lux. The SD light cycle was interrupted by a 1X30-min artificial ALAN exposure per night at 24:00 h. Light was emitted from two light fixtures (a single bulb each) that were installed one meter directly in front of the cages and half a meter apart.

Cell culture

4T1 cells originating from transgenic Balb/c mice were purchased from the American Type Culture Collection (ATCC), a global bio-source center, Manassas, VA, USA. These cells are epithelial, p53 wild type, androgen dependent and poorly differentiated [29]. Cells were grown in 25 ml or 75 ml flasks in a humidified incubator at 37 ºC with 5% CO_2 with DMEM medium supplemented with 1.5 mM L-glutamine, 2.2 g/L sodium bicarbonate, 5% fetal bovine serum and 1% penicillin-streptomycin (all from Biological Industries, Kibbutz Beit Haemek, Israel).

Experiment protocol

After the acclimation period at a given spectral composition, mice were inoculated subcutaneously (*s.c.*) into the hind flank with 1×10^6 (0.2 ml) 4T1 cells and randomly assigned to two groups (N=10 each), and one group was administered with melatonin (10 mg/L, Sigma; Saint Louis, USA) dissolved in 0.01% ethanol in the drinking water (experiment group), and the other group was only given 0.01% ethanol in the drinking water (control group). Melatonin was given during the dark period (16:00 to 08:00h) and substituted by standard sterilized tap water-containing bottles during the counterpart light period.

Mice were monitored for body mass and tumor growth twice a week for one month and thereafter sampled for urine at 4 h interval for 28 h (melatonin level analysis) and then sacrificed by CO_2 asphyxiation and tumor, lung, spleen, and liver tissues (for global DNA methylation analysis) were immediately removed and stored at –80°C until subsequent analysis. Metastasis nodules in these organs were also inspected visually and recorded. Additionally, spleen and tumor masses were recorded immediately after the mice were sacrificed (± 0.01 g; Ohaus CS200, Parsippany; USA).

Tumor growth analysis

Tumor growth was estimated twice a week by measuring the greatest tumor diameters (length and width) using a digital caliber with ± 0.01 mm accuracy (Mitutoyo, Kawasaki, Japan). Volume was calculated according to equation 1 [30]:

$$\text{Tumor volume } (mm^3) = Length * Width^2 * 0.52 \quad (1)$$

Additionally, body mass of the inoculated mice were also recorded throughout the study.

Urine collection and melatonin analysis

Urine samples were collected using a non-invasive animal method for rodents [31]. Mice were housed individually in metabolic cages (Tecniplast, SmartFlow; Buguggiate, Italy) and urine samples were collected at 4-h intervals for a period of 28-h, immediately after the tumor growth measurement. Urine volume was calculated gravimetrically in a tarred 1.5 ml Eppendorf by assuming a specific gravity of 1 g/ml [32]. All urine samples were stored at –80 °C for further analysis.

Urine samples were collected for analyzing melatonin levels by measuring 6-sulfatoxymelatonin (6-SMT) concentration; the major metabolite of the hormone in urine [33]. The quantitative determination of 6-SMT in urine was completed by a solid phase enzyme-linked immunosorbent assay (ELISA # RE54031; IBL, Hamburg;Germany) as described previously [34].6-SMT concentrations (ng/mL) were spectrophotometrically determined by ELISA microplate reader at 450 nm with reference wavelength 650 nm (PowerWave HT, Biotek, Winooski; USA) and analyzed by Gen5™ Data Analysis Software (Version 2, Biotek, Winooski; USA).

Global DNA methylation analysis

Genomic DNA was purified from tumor, lung, spleen, and liver cells using High Pure PCR Template Preparation Kit (Roche, Mannheim; Germany). Accordingly, 40 mg tissue samples were lysed at 70°C with proteinase K followed by DNA binding, washing, and eluting according to the instructions of manufacture. Global DNA methylation was evaluated using the MethylFlash™ Methylated DNA Quantification Kit (Epigentek, Farmingdal; USA) for analyzing the levels of 5-methylcytosine (5-mC). Purified DNA samples (100 ng) were incubated in 96-well plates coated with 5-mC antibody at 37°C for 90 min. After washing, a capture, antibody was loaded onto the plates and incubated at room temperature for 30 min. Thereafter, the plates were washed and an enhancer solution was added and incubated 30 min at room temperature. The percent methylated DNA was proportional to the optical density (OD) at 450 nm. DNA methylation was estimated using positive (PC; 50% 5-mC) and negative (NC; 50% unmethylated cytosin) controls according to equation 2:

$$5-mC \ (\%) = \frac{(Sample\_OD - NC\_OD)/S}{(PC\_OD - NC\_OD)X2/P} * 100 \quad (2)$$

Where S is the amount of input DNA (100 ng) and P is the amount of input PC (5 ng). The levels of methylated

DNA are calculated as percentage of the total DNA.

Statistical analysis

All statistical tests were performed using SPSS 13.0 for windows (SPSS Inc., Chicago, IL, USA). Three-way mixed repeated-measures ANOVA (RM3W-ANOVA; 2 spectral compositions X 2 melatonin X 8 time) and two-way ANOVA (RM2W-ANOVA; 2 spectral compositions X 2 melatonin) with repeated measure were used to evaluate mean value differences in urine production rates and 6-SMT levels between the studied groups. The effect of the different treatments on body mass, spleen mass, tumor growth, tumor mass, and global DNA methylation was analyzed by 2W-ANOVA for mean effects of spectral compositions (two levels) and melatonin treatment (two levels). The 2W- and 3W-ANOVA were followed by one-way ANOVA (1W-ANOVA) if effect of treatment or interactions were statistically significant. The 1w-ANOVA models were followed by Bonferroni and Tukey post hoc pairwise comparisons as appropriate. An independent Student's *t*-test was used to determined significant differences between mean levels of experimental variables (e.g., tumor growth, body mass, 6-SMT, and global DNA methylation) under the different treatments. Relations between 6-SMT, tumor growth, global DNA methylation, and relative spleen mass levels were assessed by Pearson correlation coefficient (*r*). Stepwise regression was performed to quantify relationship between 6-SMT, and both tumor volume and global DNA methylation levels.

Urine production and urinary 6-SMT levels were also analyzed for rhythmicity using the population Cosinor procedure [35]. Accordingly, the data for a given group is modeled by a non-linear regression to fit the data to the best cosine equation by successive least squares approximation to the complete data over a period of 24-h using equation 3:

$$F(t) = Mesor + Amplitude * \cos\left[2\pi * \frac{(t - Acrophase)}{Period}\right] \quad (3)$$

Where *F(t)* is 6-SMT concentration at time *t* of the best fitted equation described by *Mesor* (rhythm-adjusted mean of the best fitted curve), Amplitude (half the difference between maximum and minimum values of the best fitted curve), Acrophase (the time when the maximum levels of the measured variable occur with reference to 00:00 h), and period (24 h- length of a complete cycle). The Jankins-Watt auto-periodogram analysis was performed to estimate the period of the oscillation [36]. A rhythm is considered significant if the variances of the best fitted cosine curve and those of the linear model are not equal (F-test statistic at $P<0.05$). The population Cosinor analysis was completed by the TSA-Time Series Analysis Serial Cosinor 6.3 software (Expert Soft Technologie, Esvres; France). Data are presented as mean ± one standard error (S.E.M) or 95% confidence interval (CI) mean. The statistical error (α) was set at 5%.

Results

Body mass

The body mass of mice in all the studied groups, showed an increasing gain over the course of the experiment. Mean mass gain of mice exposed to incandescent lighting without melatonin supplement was 12.07 ± 1.05%, whereas melatonin treatment resulted in less gain. Mice under natural LED conditions, showed similar changes in body mass, but with significantly greater gains compared with those recorded in the incandescent-exposed counterpart groups (29.82 ± 1.41% and 19.98 ± 2.56% mass gains for LED and LED with melatonin, respectively; Figure 1).

Tumor growth

The duration from 4T1 cells inoculation to the appearance of a measurable tumor was designated as tumor-free interval. The tumor-free interval in the incandescent groups was five days with 100% tumor appearance, whereas only 27% of the melatonin-treated mice had visible tumor at day five while in the natural LED groups the tumor- free interval was only three days with 100% mice with visible tumor in the control group compared with only 11% in the melatonin-treated mice (Table 1).

The Tumors were evident in all mice in the different

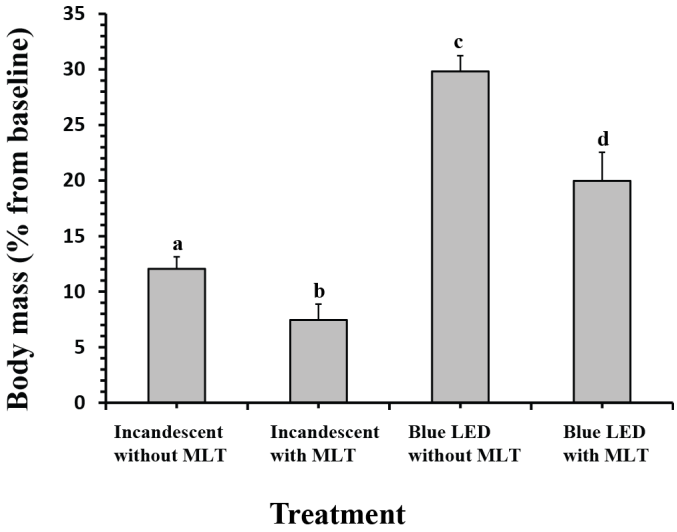

Figure 1: Percentage change in body mass of Balb/c female mice at the end of the study relative to baseline mass prior to tumor cell inoculation (1X10^6 cells). Mice were kept under short photoperiod (8L:16D, lights on from 8:00 to 16:00h) of either yellow incandescent or blue LED lightening with a single 30-min artificial light-at-night exposure per midnight for 28-days. Mice of experimental groups were nocturnally administered melatonin (10 mg/L; MLT) in the drinking water. Results are presented as mean ± S.E. of n=9-12. Different letters represent statistically significant difference among groups (Tukey's, P=0.0001).

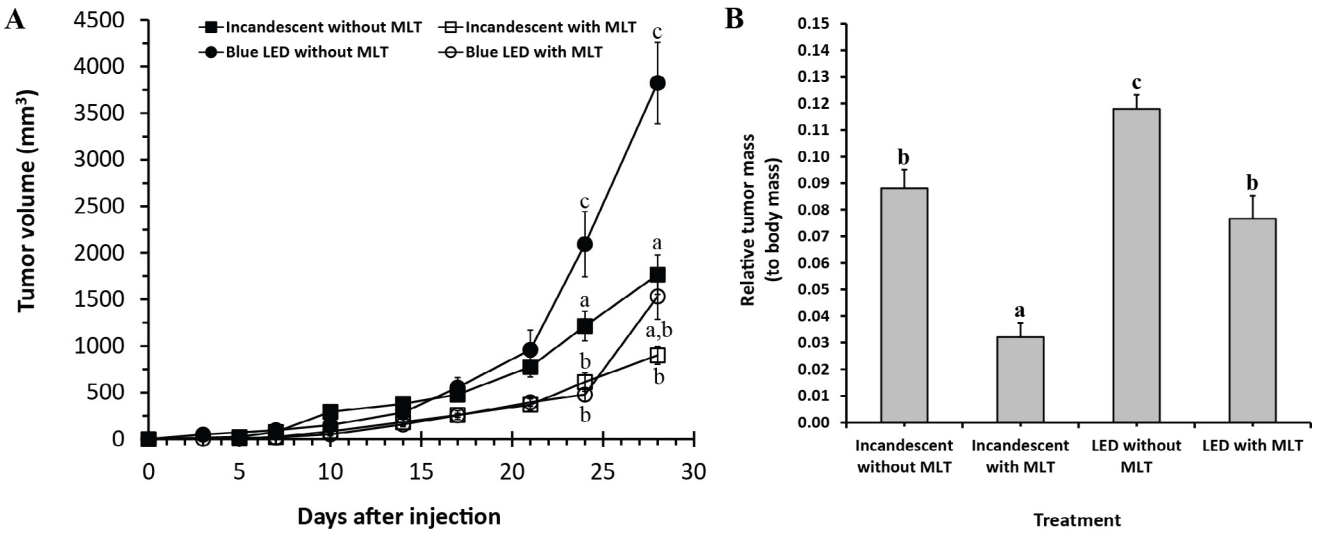

Figure 2: Tumor growth rates (A) and relative tumor mass (B) of Balb/c female mice during 4-wks post cancer cell inoculation different illumination and melatonin (MLT) treatments. Tumor volumes were measured by digital caliper semi-weekly. Tumor mass was measured on day 28 post-inoculation. Results are mean±S.E. of n=9-12. Different letters represent statistically significant difference among groups at the same day (Tukey's, P=0.0001).

Table 1: Effect of different spectral compositions combined with melatonin (MLT) treatment on tumor and metastasis developments in Balb/c female mice.

Treatments*	Tumor volume (mm^3)	Metastasis** (%)
Yellow without MLT	1766 ± 244[a]	50
Yellow with MLT	900 ± 96[b]	0
Blue without MLT	3453 ± 309[c]	89
Blue with MLT	1532 ± 245[a,b]	44

*- Measurements were performed at 4-wks post tumor cell inoculation.
**- Percent of mice that had metastasis to the lung, liver, or spleen as was inspected visually after 4-wks.
Different letters represent statistically significant difference among groups (Tukey's, P<0.01).

Table 2: Population Cosinor analysis of urine production rates in Balb/c female mice inoculated with 4T1 breast cancer cells under short day photoperiod of yellow incandescent and blue LED illuminations with artificial light-at-night.

Treatment	Period (h)	Mesor (µL•g-1•h-1)	Amplitude (µL•g-1•h-1)	Acrophase (hh:min)	PR* (%)	p**
		[95% Confidence interval of the group mean]				
Yellow without MLT	16.3	1.77 [1.36; 2.18]	0.53[a] [0.49; 6.94]	10:19 [09:31; 11:06]	37	0.03
Yellow with MLT	18	1.71 [1.36; 2.07]	0.89[b] [0.34; 1.45]	08:06 [06:00; 10:09]	34	0.004
Blue without MLT	24	1.51 [1.10; 1.92]	0.30[a] [0.15; 0.44]	06:56 [04:40; 09:12]	37	0.01
Blue with MLT	8.6	1.46 [1.12; 1.79]	0.50[a] [0.35; 0.69]	05:17 [04:58; 05:34]	45	0.04

Values in brackets for Mesor, Amplitude, and Acrophase indicate 95% confidence intervals of the group mean.
*- Percentage of the rhythm represents the proportion of the total variance of data accounted for by the Cosinor procedure.
**- P-value for the F-statistic that the amplitude of the rhythm differ from zero.
Different letters indicate significant differences between groups (Tukey's, P<0.01).

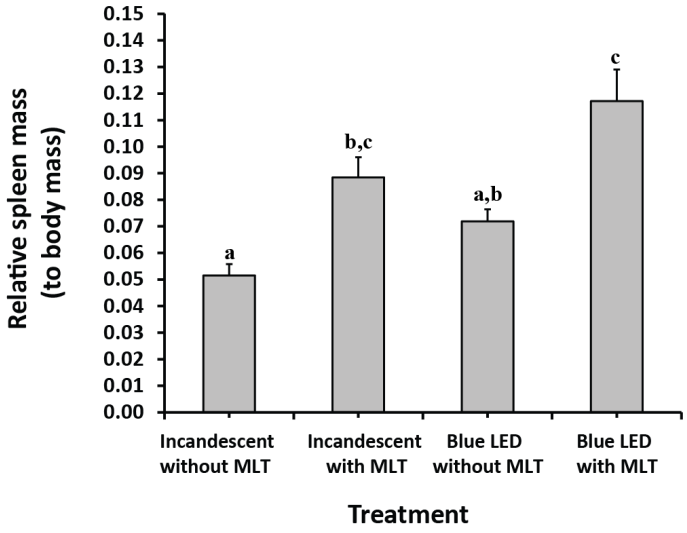

Figure 3: Spleen mass presented as a percentage of body mass, in Balb/c female mice 4-wks after breast cancer inoculation under different illumination conditions (incandescent and natural LED) and melatonin (MLT) treatment. Results are mean ± S.E. of n=9-12. Different letters represent statistically significant difference among groups (Tukey's, P=0.0001). For further details, see legend for Figure 1.

Table 3: Population Cosinor analysis of 6-sulfatoxymelatonin concentrations of Balb/c female mice inoculated with 4T1 breast cancer cells under short day photoperiod of yellow incandescent and blue LED illuminations with artificial light-at-night.

Treatment	Period (h)	Mesor (µL•g-1•h-1) [95% Confidence interval of the group mean]	Amplitude (µL•g-1•h-1)	Acrophase (hh:min)	PR* (%)	p**
Yellow without MLT	24	0.45[a] [0.39; 0.50]	0.17 [0.02; 0.33]	23:36 [21:04; 02:08]	41	0.05
Yellow with MLT	24	3.85 [3.69; 4.00]	0.16 [0.04; 0.35]	22:20 [18:32; 02:11]	35	0.34
Blue without MLT	24	0.19[b] [0.16; 0.21]	0.14 [0.08; 0.20]	21:52 [19:28; 00:12]	52	0.02
Blue with MLT	24	4.47 [4.26; 4.68]	0.06 [0.15; 0.28]	06:04 [00:20; 11:36]	25	0.66

Values in brackets for Mesor, Amplitude, and Acrophase indicate 95% confidence intervals of the group mean.
*- Percentage of the rhythm represents the proportion of the total variance of data accounted for by the Cosinor procedure.
**- P-value for the F-statistic that the amplitude of the rhythm differ from zero.
Different letters indicate significant differences between groups (Tukey's, P<0.01).

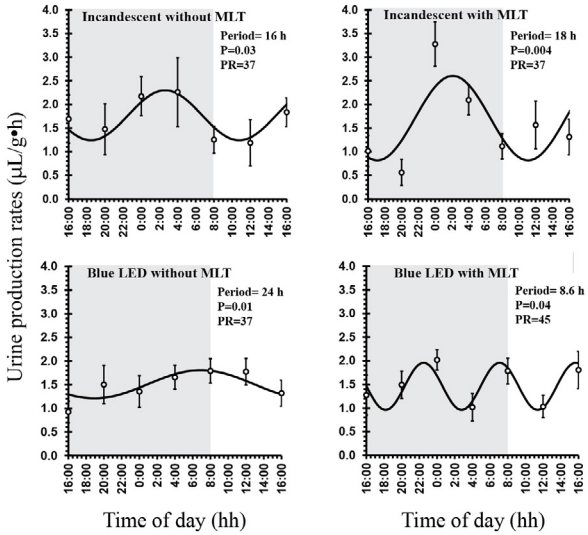

Figure 4: Urine production daily rhythms rates of Balb/c female mice 4-wks after acclimation under two illumination conditions (incandescent and natural LED) with and without melatonin (MLT). Results are mean ±S.E. of n=9-12. The best-fitted cosine curve and Cosinor estimates (period, P-value, and percentage of the rhythm [PR]) are depicted. Gray area in each plot represents the length of the dark period. For further details, see legend for Figure 1.

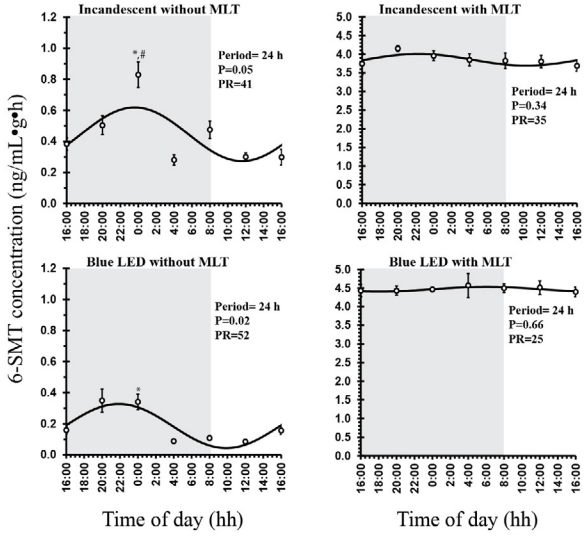

Figure 5: 6-sulfatoxymelatonin daily rhythms of Balb/c female mice under two different illumination conditions (incandescent and natural LED), with and without melatonin (MLT) treatment, after 4-weeks of acclimation.. Results are mean ± S.E. of n=8. The best-fitted cosine curve and Cosinor estimates (period, P-value, and percentage of the rhythm [PR]) are depicted. Gray area in each plot represents the length of the dark period. * P=0.03 *vs* 12:00h and # P=0.0001 *vs* Blue without MLT (*t*-test).

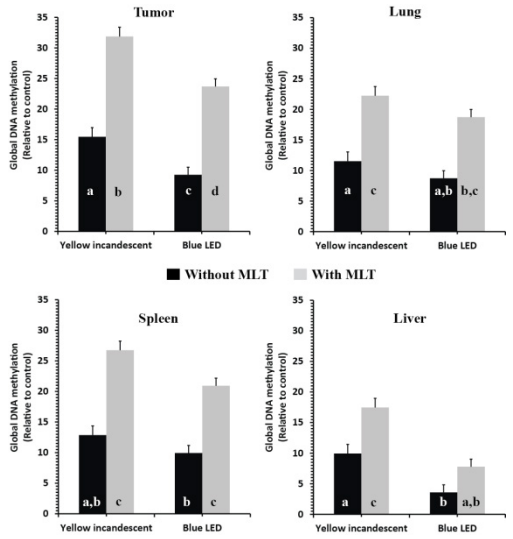

Figure 6: The effects of spectral composition and melatonin (MLT) levels on global DNA methylation in Balb/c female mice after 4-weeks acclimation under experimental conditions. Results are mean ± S.E. of n=8. Different letters represent statistically significant difference among groups within the same tissue (Tukey's, $P<0.05$). For further details, see legend for Figure 1.

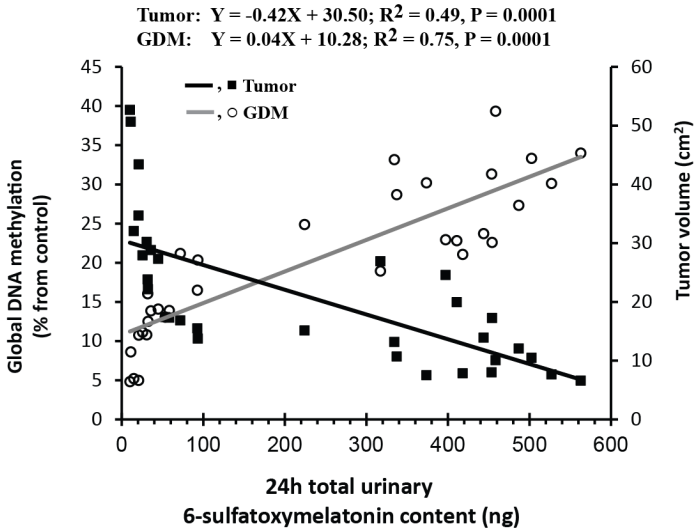

Figure 7: Correlation between daily urinary 6-sulfatoxymelatonin contents with both tumor global DNA methylation (GDM) and tumor volume in Balb/c female mice after 4-wks acclimation under experimental conditions. The regression analysis was imposed on the combined data of all groups in the study (N=32). Black and gray solid lines represent the regression lines. The estimated equation, R^2, and P-value are also depicted. For further details, see legend for Figure 1.

treatment groups at day 10 after the inoculation. The RM3W-ANOVA model detected significant time ($F_{7,252}$=80.93, P=0.0001), spectral ($F_{1,36}$=5.31, P=0.03), and melatonin ($F_{1,36}$=25.25, P=0.0001) effects on mean tumor growth rates (Figure 2A).

Tumor development in all the studied groups, demonstrated rapid growth rates particularly after 15 days from inoculation. Nonetheless, significant differences in tumor growth rates were recorded only after 20 days from inoculation, in which melatonin-treated mice under both spectral compositions showed significant reduced growth rates in comparison with untreated mice. Mean tumor volume on day 28 post inoculation was larger for the natural LED-treated mice without melatonin (3453 ± 309 mm^3) versus values in the other treatment groups (Table 1). Similarly, relative tumor mass to body mass was significantly greater for natural LED-treated mice without melatonin (0.12 ± 0.005%) than for the other treatment groups (Figure 2B; 1W-ANOVA, $F_{3,37}$=26.04, P=0.0001). Melatonin treatment significantly (p<0.01) reduced relative tumor mass in both spectral compositions compared with untreated mice. The incandescent mice with melatonin had the lowest relative tumor mass (0.3 ± 0.005%) compared with the other tested groups. We also recorded percent of mice that had metastasis to lung, liver, and spleen. Breast cancer 4T1 cells aggressively metastasized to lung, liver, and spleen in mice exposed to incandescent (50%) and natural LED (89%) wavelengths. Melatonin supplement in the drinking water clearly decreased metastasis formation in both incandescent - and natural LED-treated mice (0%, and 44%, respectively) compared with counterpart controls (Table 1).

The 2W-ANOVA model revealed significant effect of wavelength ($F_{1,37}$=10.04, P=0.003), and melatonin treatments ($F_{1,37}$=25.58, P=0.0001) on the relative spleen mass, but no interaction effects between wavelength and melatonin was detected ($F_{1,37}$=0.32, P>=0.05). Melatonin supplement during the night significantly increased relative spleen mass under both wavelengths compared with controls (Figure 3).

Urine production rates and 6-SMT levels

Daily rhythms in urine production rates are presented in Figure 4. The Cosinor analysis detected significant urine output daily rhythms for all the studied groups, but with different periods ranging from 8.6 h to 24 h. Mesor levels were not changed by neither wavelength nor melatonin treatments, however amplitude levels in melatonin-treated mice under incandescent exposure (0.89 µL/g*h) were significantly (P<0.01) higher compared with the other experimental groups. Acrophases of the different experimental groups occurred at the second half of the dark period, except for mice exposed to incandescent wavelength without melatonin treatment (10:19 hh:min; Table 2).

All the tested groups exhibited a typical nocturnal increase in melatonin levels as reflected by its major urinary metabolite 6-SMT with oscillation over a period of 24 h (Figure 5). However, the 24 h daily rhythms were significant for the two spectral groups that were not melatonin-treated. The 2W-ANOVA analysis detected significant time ($F_{1,14}$=9.61, P=0.008) and wavelength ($F_{1,14}$=97.89, P=0.0001) effects on urinary 6-SMT, but no significant timeXwavelength interaction effects ($F_{1,14}$=0.34, P=0.57). Mice exposed to incandescent lighting had significantly higher mean levels of melatonin when compared with mice exposed to natural LED (0.44 ± 0.02 ng/ml*g*h and 0.18 ± 0.01 ng/ml*g*h, respectively). Tumor bearing-mice exposed to incandescent lighting had elevated mesor levels (0.45[0.93;0.5 ng/ml*g*h] compared with natural LED mice (0.9[0.16,0.21] ng/ml*g*h). However, no significant differences were detected for amplitude and acrophase occurrence in tumor-bearing mice under exposure to both incandescent and natural LED lighting (Table 3).

Global DNA methylation levels

A melatonin-related hypermethylation was observed in all tissues under the two wavelengths compared with hypomethylation in mice that were exposed to ALAN but without melatonin treatment (Figure 6; 2W-ANOVA: Tumor $F_{1,28}$=126.12, P=0.0001; Lung $F_{1,28}$=19.82, P=0.0001; Spleen $F_{1,28}$=28.49, P=0.0001; Liver $F_{1,28}$=14.15, P=0.0001). In tumor tissues, melatonin

supplement increased mean global DNA methylation levels to 31.87 ± 1.5% and 23.69 ± 1.24% compared with 15.46 ± 1.28% and 9.26 ± 1.45% in mice exposed to incandescent and natural LED wavelengths without melatonin, respectively. Significant wavelength-related changes in mean global DNA methylation were detected only for tumor and liver tissues (2W-ANOVA: Tumor $F_{1,28}$=27.4, P=0.0001; Liver $F_{1,28}$=24.83, P=0.0001). Under incandescent lighting exposure, higher methylation levels were found in both tumor and liver tissues compared with natural LED mice (Tumor: 15.46 ± 1.28% and 9.25 ± 1.45%; liver: 9.72 ± 1.59% and 3.57 ± 0.81, respectively).

Correlation analysis

In order to maximize statistical power of the correlation test, we combined the data for each tested variable from the different experimental groups together to be analyzed as a single data set. The Pearson's correlation analysis revealed a positive correlation between mean 6-SMT levels and mean relative spleen mass (r=0.66, N=32, P=0.0001), but no significant correlation was detected between relative tumor volume and relative spleen mass (r=-0.21, N=32, P>0.05). The mean levels of 6-SMT exhibited a negative (r=0.7, N=32, P=0.0001) and positive (r=0.87, N=32, P=0.0001) correlation with the levels of tumor volume and global DNA methylation levels, respectively (Figure 7). Additionally, we revealed a strong negative correlation between mean global DNA methylation levels and mean tumor volume (r=0.82, N=32, P=0.0001).

Discussion

Increasing light pollution produced by excessive use of artificial light-at-night (ALAN) has become a serious environmental and public health concern [28]. Generally, most of the evidence connecting between ALAN and cancer risk is circumstantial or indirectly suggested from epidemiological studies showing an association between ALAN and increased cancer incidences in modern societies [3]. Our results suggest that ALAN of short-wavelength is an effective stimulus for breast cancer cell proliferation and support the suggestion that ALAN coincide with increased risk of cancer.

In several epidemiological studies, it has been repeatedly suggested that ALAN exposure is associated with breast cancer incidences particularly among women how work at night [18,19]. Furthermore, this association also suggested to be characterized by a direct dose-dependent relation between degree of exposure (years of employment and the cumulative shifts involved) and breast cancer risk [4,5]. In nude rats bearing human breast cancer xenograft, ALAN exposures resulted in rapid tumor growth compared with tumor-bearing rats under control conditions [10]. Recent research from our laboratory has shown that ALAN from cool white florescent (450 lux and 469 nm) accelerated tumor growth rates in 4T1 inoculated Balb/c mice when compared with controlled short day 4T1 inoculated mice, which were not exposed to ALAN [11].

ALAN exposure inhibits key enzymatic pathway in melatonin biosynthesis and suppress further nocturnal increase in the hormone levels [37]. Although, melatonin suppression is sensitive to broad range of spectral compositions [38,39], short-wavelengths (420nm -520nm) are the most potent inhibitor of melatonin production [40]. Our results support the negative dose-dependent relation between wavelength and melatonin suppression as they showed that natural LED lighting (460 nm) is more than two times effective in suppressing mean melatonin levels compared with incandescent lighting (580 nm). These differences in melatonin suppression, can at least partly describe the LED-induced higher tumor growth rates when compared to rates under incandescent lighting exposure. Nocturnal melatonin supplement in drinking water for 28 days in both incandescent and natural LED ALAN-treated 4T1 inoculated-mice, significantly reduced tumor volume and metastasis formation compared with control tumor bearing-mice.

The oncostatic effect of melatonin on tumor development is well recognized [41]. In a case-control study among postmenopausal women, a significant inverse relation between urinary 6-SMT and breast cancer risk was reported [42]. In this study, the follow-up of a cohort of 3966 women reported that fewer women with high 6-SMT levels develop breast cancer during the follow-up than women with low 6-SMT levels. The

supportive evidence for the anti-carcinogenic property of melatonin comes also from experimental studies in animal models [43]. In mice bearing Ehrlich and Fibrosarcoma tumors, melatonin treatment inhibited tumors growth and lengthen survival compared with controls, and these effect were related to photoperiod and time of melatonin administration [44]. Similar oncostatic effects of melatonin were also reported in rats whereas pinealectomy increased mammary tumor development and melatonin treatment significantly decreased tumor incidence from 79% to 20% compared with untreated rats [45,46]. White florescent ALAN with increasing irradiances suppressed melatonin production and advanced tumor growth in both hepatomas and breast cancer xenograft tumor-bearing rats in a dose-dependent pattern and these effects were antagonized when tumors were perfused with nocturnal blood drawn from premenopausal women containing physiological levels of melatonin [47].

Here we provide supportive evidence for the oncostatic effect of melatonin under short - and long-wavelengths emitted from natural blue-white LED and incandescent lightings. In 4T1 inoculated mice, the tumor growth was more aggressive in response to natural LED ALAN than the counterpart incandescent exposure. Nocturnal melatonin administration efficiently antagonized the ALAN-induced tumor development in both natural LED - and incandescent exposed inoculated mice, but the effect was less pronounced under the LED conditions.

Melatonin can constrain cancer cell proliferation by regulating, sex hormone productions [48], cancer metabolism [49], cell apoptosis [50], cancer-related stress and immune functions [51], and oncogene expressions [52]. DNA methylations regulated by melatonin are a promising mechanism linking between environmental exposures (e.g., ALAN) and cancer progression. These reversible modifications can directly regulate a wide array of gene expressions involve in promoting carcinogenic activities such as tumor cell proliferation, metastasis formation, and estrogen-related responses [53]. In this study, global DNA methylation in control tumor-bearing mice (without melatonin treatment) showed tissue-specific spectral responses with lower global DNA methylation levels in tumor and liver tissues collected from natural LED-inoculated mice compared with levels detected in the same tissues for incandescent exposed mice. Nevertheless, mean methylation levels were similar in lung and spleen tissues under the two spectral compositions. Furthermore, we revealed a negative correlation between tumor growth rates and melatonin levels, whereas global DNA methylation levels varied in direct relation to the level of melatonin.

Our results are in agreement with previous studies reporting an inverse relationship between melatonin levels and tumor development in humans [42] and rodent models [43] and further support the therapeutic feature of melatonin in the treatment of tumors, such as breast cancer. The involvement of epigenetic modification in tumor etiology, including breast cancer is well-established [25]. The genome of breast cancer is generally characterized by global hypomethylation [54] and specific promoter hypermethylation of proto-oncogenes [55] involved in regulating mitogenic activity. These aberrant methylations are recurrently associated with genomic instability, over expression of oncogenes (e.g. *breast cancer gene 1*), silencing tumor suppressor genes, uncontrolled cell proliferation, and up-regulation of metastatic genes, all of which promote cancer progression [26,56]. Melatonin was shown to be involved in silencing breast cancer-related oncogenes by methylation of the estrogen synthetase or deacetylation of the enzyme histone [57]. In human breast cancer cell lines, melatonin perfusion induced DNA hypermethylation that was associated with low expression of the oncogenes EGR3 and POU4F2/Brn3b and increased expression of the tumor suppressor gene GPC3 [52]. Finally, the melatonin-induce hypermethylation in 4T1 inoculated mice here is in agreement with the above reported concurrent, but the exact mechanism of action by which the hormone modulate epigenetic responses needs further to be studied.

Finally, our results demonstrate that the relative spleen mass in 4T1 inoculated mice was affected by the exogenous melatonin treatment with a significant positive correlation with the hormone levels. Melatonin treatment moderately elevated the relative mass of the spleen in both unstressed active and passive rats [58]. Furthermore, spleen size was much higher in skin tumor-bearing mice than in healthy animals [59]. Generally,

melatonin is a potent immune-modulatory agent for boosting immune responses particularly inflammation and to short-day photoperiod that represent the challenging conditions of the winter season [60,61]. The stimulating function of melatonin on the immune system is likely to explain the increased relative spleen mass in 4T1 inoculated mice.

Conclusions

Presently, the LED technology is increasingly invading virtually every aspect of our modern lifestyle including public and domestic applications [62]. The new technology is highly promising in regards to efficiency, power and cost when compared with traditional systems such as fluorescent and incandescent illuminations [63]. However, intensive use of LED lighting is expected to dramatically increase wavelength emission at the short end of the visible spectrum, close to the peak spectral sensitivity (469 nm) of melatonin suppression (16). Currently, there is compelling evidence from epidemiological and experimental studies that excessive exposures to ALAN are associated with suppressed melatonin levels and increased cancer incidences. Our results provide strong support for this association and show for the first time that exposure to natural blue-white LED during the night encloses higher potential to stimulate cancer development than the traditional incandescent lighting. As a modern society with inevitable extended nocturnal activity, we should promptly act to promote countermeasures for reducing the negative impacts of short-wavelengths ALAN by developing new light technologies and/or refining existing ones with improved efficiency and enhanced safety for human. Environmentally friendly illumination designs should be at least filtered for short-wavelength as those emitted here by the natural LED lighting, before been introduced to the market.

Our results support the following nexus ALAN, pineal melatonin suppression, Global DNA methylation, 4T1 tumor growth presumably by increasing proliferation [27]. Therefore, it is also suggested that epigenetic modifications, particularly DNA methylation can provide a mechanism of action linking between environmental exposures (e.g., ALAN) and cancer activities. Bearing in mind that epigenetically modifications are reversible in regards to breast cancer it opens new ways for treating breast cancer patients. Melatonin and DNA methylation profiling are promising and powerful tools as biomarkers for cancer development and therapeutic agents for targeting signaling pathways in malignant cells.

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Gratis

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