

# Effects of chronobiology on prostate cancer cells growth *in vivo*

## Efeitos da cronobiologia no crescimento de células cancerígenas da próstata *in vivo*

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### ABSTRACT

**Background and objective:** The increased risk of developing prostate cancer (PC) observed in recent decades in industrialized countries has showed to be related at least partially to the elevated exposure to artificial light at night and to long photoperiod during all the year. However, the precise effects of light and photoperiod manipulations on prostate cancer cell proliferation *in vivo* have not been reported to the same extent. **Methods:** We exposed male C57BL/6 mice to short or long photoperiods (short day, SD, and long day, LD). After inoculation of TRAMP-C2 cells, half of the SD mice were also exposed to light interference at night while half of the LD mice were treated with melatonin. **Results:** Under LD-acclimation, tumours were significantly larger compared to SD conditions. Melatonin treatment to LD mice reduced tumour size significantly, while light interferences to SD mice tended to increase it. **Conclusions:** we conclude that exposure to LD and light interference may promote cancer growth via changes in melatonin production and secretion. Our results strongly support a novel link between temporal variables and cancer incidence. Accordingly, we anticipate our findings to increase the awareness of scientists as well as health officials and policy makers to the adverse effects of illumination misuse during the night.

**Keywords:** Light; Circadian rhythm; Climate; Sunlight; Prostatic neoplasms; Environment; Melatonin; Photoperiod; Chronobiology disorders

### RESUMO

**Introdução e objetivos:** O aumento de exposição à luz artificial durante a noite e, portanto, aumento do foto-período anual, tem sido apontado como fator de risco, mesmo que parcial, para o crescimento da ocorrência de risco de câncer da próstata (CP) observado nas últimas décadas em países industrializados. Entretanto, apesar das evidências, os efeitos da luz e da manipulação do foto-período sobre a proliferação *in vivo* de células cancerígenas da próstata não têm sido descritos com exatidão nos estudos que abordam o tema. **Métodos:** Camundongos machos C57BL/6 foram expostos a períodos luminosos curtos (PLC) e longos (PLL) de luz. Após a inoculação destes animais com células TRAMP-C2, metade dos camundongos PLC foi exposta à interferência luminosa durante a noite, enquanto a outra metade, dos camundongos PLL, recebeu tratamento com melatonina. **Resultados:** Ficou evidente que, durante a aclimação, os tumores dos animais PLL eram significativamente maiores do que os de animais PLC. O tratamento com melatonina em animais PLL reduziu o tamanho dos tumores de

forma significativa, enquanto a interferência luminosa em animais PLC levou a uma tendência ao aumento de tamanho. **Conclusões:** É possível que PLL e interferência luminosa possam promover o crescimento de células cancerígenas da próstata através de alterações na síntese e secreção de melatonina. Nossos resultados evidenciam uma associação desconhecida entre variáveis temporais e a incidência de câncer. Com isto, esperamos que esta descoberta sensibilize pesquisadores, profissionais e legisladores que atuam na Saúde Pública com relação os efeitos negativos que a luminosidade noturna tem sobre o organismo.

**Descritores:** Luz; Ritmo circadiano; Clima; Luz solar; Neoplasias da próstata; Meio ambiente; Melatonina; Fotoperíodo; Transtornos cronobiológicos

### INTRODUCTION

Seasonality is a basic temporal environmental variable to which organisms are adapted. Such adaptation includes: anatomical, physiological, biochemical, immune function and behavioural pattern features. Outside of the tropical photoperiod changes are the initial and the most important environmental cue for seasonal acclimatization of such features. In mammals, photoperiod signals are transferred to tissues and cells by the neurohormone melatonin (MLT) produced and secreted from the pineal gland during the dark phase of the 24-hour cycle. An increase in plasma MLT levels for an extended duration is a signal for winter, while a decrease in levels and secretion duration signals the incoming summer. Like other animals, humans are seasonal in birth rates, mortality, suicide rates, and many more <sup>(1,2)</sup>.

The invention of the incandescent light bulb by Thomas Edison some 130 years ago led to a dramatic change in human life style, which spread throughout the entire world during the 20<sup>th</sup> century. Light at night (LAN) was of great importance in changing human social and behavioural habits towards an expansion of their waking hours, thus resulting in an extended exposure to illumination during the hours when human ancestors have typically been in the dark over millions of years of primate evolution. LAN has also impacted on daily rhythms, as light is the main zeitgeber of the internal

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biological clock. The exposure to LAN, therefore, leads to a disruption of the daily rhythms, particularly if it occurs at the middle of the night. Since illumination has practically abolished seasonality and disrupted the daily rhythm schedules, it is worth asking: what are the implications, if any, of this change in temporal environment on human health?

The prevalence of prostate cancer (PC) has increased significantly in the last decades and became the second main cause of death among men in many industrial countries, constituting about 33% of the diagnosed cancers in males, and it is estimated that 50% of the diagnosed patients will develop metastases<sup>(3)</sup>. In recent years, the PC risk is of great concern not only in developed countries but also in developing countries. Environmental changes are considered as a factor possibly responsible for the increase in PC incidence. Can environmental temporal changes, emerging from illumination and the avoidance of daily rhythm and seasonality, be responsible at least in part for such increase in PC incidence?

A recent study carried out on a global scale<sup>(4)</sup> revealed an increase in the risk of PC incidence with the increase in LAN, whereas the incidence of lung or colon cancers was not affected. These results suggest that changes in photoperiod caused by LAN, which decreases the number of dark hours as well as pineal MLT secretion, an anti-oncogenic agent, can also be a cause for the increased PC incidence in the heavily illuminated countries. We suggest the following hypothesis: "if MLT is an anti-oncogenic agent then in mice acclimated to short day (SD) conditions the proliferation of PC cells will be slower than in long-day (LD)-acclimated mice". Moreover, MLT treatment to LD-acclimated mice should have a similar effect as SD acclimation, while light interference (LI)-exposed mice should present an effect similar to LD. The objective of our study was to test this hypothesis in mice under *in vivo* conditions by exposing inoculated mice with cancer cells to four different conditions: 1) LD, 16L:8D; 2) SD, 8L:16D; 3) LD and MLT-treatment; 4) SD and LI.

## METHODS

### Animal maintenance

Male mice C57BL/6, five weeks of age were purchased from Harlan Laboratories (Jerusalem, Israel). The animals were housed in polycarbonate cages (4–6 mice/cage) and maintained at room temperature of  $25 \pm 1^\circ\text{C}$ . Mice were fed (rat pellets, Koffolk 1949, purchased from Koffolk, Inc., Tel Aviv, Israel) and given tap water *ad libitum*. All procedures were conducted with approval of Haifa University Institutional Animal Ethics Committee and the ministry of Health, Israel. In regards to MLT production by C57BL/6 mice, please see discussion.

### Effect of photoperiod, LI and MLT on tumour development

Cool white fluorescent illumination (at an intensity of 450 lux and dominant wave length of 469 nm) was provided during

photophase and red dim light (intensity of 25 lux and dominant wave length of 680 nm) during scotophase. Thirty-six mice were randomly assigned to the following photoperiod regimes for an acclimation period of four weeks prior to inoculation: LD (16L:8D), lights were on between 8 and 24h, (n=18). SD (8L:16D), lights were on between 8 and 16h, (n=18). TRAMP-C2 cells were suspended at a concentration of  $3 \times 10^7$  cells/mL medium. Aliquots of 0.1 mL ( $2 \times 10^6$  cells) were injected subcutaneously into the mouse hind dorsal part, using a 27-gauge needle. Following inoculation, each group was divided into two subgroups (n=9 each). LD mice were divided into melatonin treated (MLT mice) and control (untreated). For the first two weeks, 0.2 mL MLT, resuspended in 7% ethanol (M5250, Sigma-Aldrich, Rehovot, Israel), were injected intraperitoneally every day, six hours before lights went off ( $5 \text{ mg/kg.W}_b$ ), whereas during the rest of the experiment, MLT was offered in the drinking water ( $10 \text{ mg/kg.W}_b$ ) beginning six hours before lights went off until the end of the dark period. Tap water was offered to the mice at the rest of the light period. SD mice were divided into control and light interfered (LI mice). The latter ones were exposed every day, seven hours after lights went off to 30 min of light (450 lux and a dominant wave length of 469 nm). Mice body mass ( $W_b$ ) was measured using semianalytical scale (1907 MP8 Sartorius, Germany) throughout the experiment, which lasted for 59 days post-inoculation. Tumour size was measured twice a week using a calliper and the volume was calculated by the formula: length x width<sup>2</sup> x 0.52<sup>(5)</sup>.

## Statistics

All data are expressed as mean  $\pm$  standard deviation (SD). A two-way ANOVA analysis was used for testing all photoperiod treatments, while Student's t-test was used for comparing results within a photoperiod (SD or LD) given group. For this analysis, we used the SPSS 12.0 software.

## RESULTS

### Effect of photoperiod, LI and MLT on PC tumour growth

Tumours first appeared after about three weeks. Significant ( $p < 0.001$ ) differences in tumour volumes were observed between LD and Short Day mice at and beyond 36 days post-inoculation of TRAMP-C2 cells. At 59 days post-inoculation, the average tumour volume was  $5.92 \pm 2.07 \text{ cm}^3$  in LD mice and only  $0.85 \pm 0.41 \text{ cm}^3$  in Short Day mice, indicating a faster growth rate in the former group (Figure 1A). In LD mice treated with MLT, the average tumour volume at day 59 was  $0.62 \pm 0.14 \text{ cm}^3$ , significantly ( $p < 0.001$ ) smaller than in control untreated LD mice (Figure 1B). Survivorship at day 59 post-inoculation was 55.5% (five out of nine) in the MLT-treated group as compared to only 33.3% (three out of nine) in the untreated group. Relative to the Short Day mice, significant increases ( $p < 0.05$ ) in tumour volumes were detected in LI mice;

tumour volumes were  $0.85 \pm 0.41 \text{ cm}^3$  and  $1.84 \pm 0.25 \text{ cm}^3$ , respectively (Figure 1C). There were no differences in survivorship (44%) between the two groups.

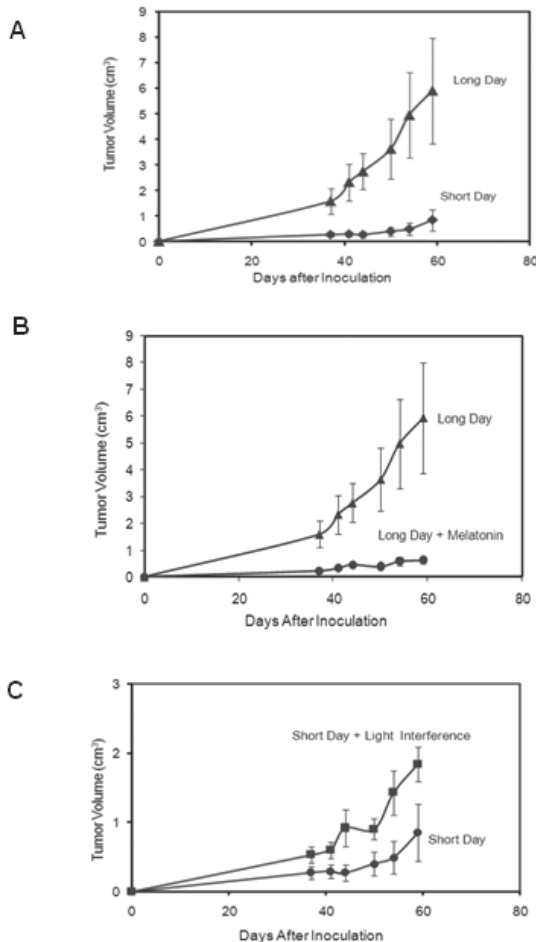
## DISCUSSION

For millions of years, humans and their ancestors were limited in their activities by the unavailability of night-time lighting. Although fires, candles and oil lamps allowed our predecessors to continue some functions after dark, these light sources lacked

the power and the universal access to erase the difference between night and day. As a result, for >>99% of humans history, people have generally wound down at night. This has changed dramatically with the introduction of illumination into modern human life, which has brought much prosperity and transformed our lifestyle forever. However, similarly to many of the technologies that we have developed to help with our daily lives, this change also ran against the grain of millions of years of evolution. Over the last two decades, the results of numerous studies that questioned possible impacts of LAN and LI in animal models revealed the potential harmful interruption of circadian rhythms, as well as the interference with seasonality, a phenomenon named “seasons out of time”<sup>(6)</sup>. Additionally, LI was shown to be a stressor in social voles *Microtus socialis*<sup>(7)</sup>. Humans all over the world are exposed to a new environment in which the temporal variables were changed, abolishing daily rhythms and seasonality. LAN, with all its economical blessing, is now considered as a source of pollution<sup>(8-10)</sup>. One important outcome of this awareness is reflected by the recent decision of the International Agency for Research on Cancer (IARC), the cancer arm of the World Health Organization, to classify over-night shift work as a probable carcinogen factor<sup>(11)</sup>.

The results of the current study can be explained based on the progress made in our understanding of the way that photoperiod changes are transmitted to the cells, the function of the system as a clock (daily rhythms) and as a calendar (seasonality), and the important role of pineal MLT production and secretion. Melatonin is a hormone that controls several important functions and shows strong anti-oxidative and anti-oncogenic effects<sup>(12)</sup>. Preliminary results of *in vitro* experiments carried out in our laboratory showed that MLT inhibited the proliferation of C57BL/6 derived prostate cancer cells (TRAMP-C) (Yukler, A. Unpublished data, 2008). These results suggest that TRAMP-C cells may contain MLT receptors. The mice that we used for our experiments, C57BL/6, were considered as natural MLT ‘knockdown’ mice<sup>(13)</sup>. However, a low but significant increase in MLT synthesis could be observed in C57BL/6 pineal gland upon norepinephrine stimulation, and, notably, also when animals were exposed to long nights. The authors thus concluded that the commonly used C57BL/6 mice are not completely MLT-deficient<sup>(14)</sup>.

In LD-acclimated mice, as comparing to SD-acclimated mice, MLT levels are assumed to be lower. This might explain the difference in tumour growth rate between the two groups. In this sense, treatment with MLT to LD mice mimics exposure to SD. In accordance, MLT-treated LD mice demonstrated a significant decrease in the tumour growth. Low levels of MLT were previously measured in C57BL/6 mice under LD conditions of 14L:10D<sup>(13)</sup> and this could be similar to the LD group in our experiments, but not to the SD group, in which MLT levels could be higher. LI given to



**Figure 1:** Growth rate of TRAMP-C2 cells ( $2 \times 10^6$ ) *s.c.* inoculated into the posterior dorsal part of male C57BL/6 mice. A: Effect of photoperiod - comparison between long day exposed mice (LD, triangles) or short day (SD, diamonds) as described in materials and methods. B: Effect of Melatonin (MLT) administration. Animals were exposed to LD with MLT (circles) or control (triangles) as described in materials and methods. C: Effect of Light interference (LI). Animals were exposed to short day (SD, circles) or SD with LI (squares) as described in materials and methods. All mice were followed for the development of tumours for 59 days post inoculation. Results are expressed as tumour volume ( $\text{cm}^3$ )  $\pm$  SD. \* Different scale, compared to A and B (up to  $3 \text{ cm}^3$ ), was used in figure C to plot tumour volume against days after inoculation. In B, standard deviation values for tumor volume under LD + MLT (circles) conditions are too small to be seen in the figure.

SD mice should presumably reduce pineal MLT secretion, thus exposing the mice to MLT levels similar to LD conditions, resulting in an increase of tumour size.

Results of studies on the photoreceptors in the mammalian retina revealed the existence of non-image forming photoreceptive cells (ipRGC) which contain melanopsin, a photopigment that is stimulated by light across the visible spectrum, but with a maximum sensitivity in the blue range. The axons from the ipRGCs join to form the Retino Hypothalamic Tract that innervates the Suprachiasmatic Nuclei (SCN), and from the SCN sympathetic nerves transfer the information to the pineal gland<sup>(15)</sup>. The dark period of the 24-hour cycle is the signal for MLT production and secretion by the pineal gland, even for mice with natural MLT 'knockdown'<sup>(13,14)</sup>. Although the inhibitory effect of exogenous MLT seems to suggest it plays a role in photoperiodic manipulation of tumour size, the exact underlying mechanism is largely unknown. Another possible mechanism underlying LI effect on tumour growth might be via stress response. This hypothesis is supported by previous data, demonstrating light interference as a stressor<sup>(7)</sup>. Elevated levels of stress hormones may also have an effect on cancer cells proliferation. Both mechanisms can act together, as MLT might affect (suppress) tumour growth in the LD mice, but LI affect (accelerate) tumour size via other mechanisms, including stress response.

Environmental variables can affect organisms through epigenetic changes of DNA methylation patterns and/or modification of histones in the chromatin. Among others, such changes are proposed to be a cause for silencing tumour suppressor genes<sup>(16)</sup>. Furthermore, if MLT may have the potential to modify such changes, then suppression of MLT production by LAN or LI can lead to silencing of tumour suppressor genes and, thus, promote cancer. If this is the case, then LD acclimation, in modern life style, reduces MLT production and may possibly be the reason for PC incidence increase in men in the industrialized world, as recently shown by some authors<sup>(14)</sup>. The bright side is that since this could be an epigenetic effect it can be reversed. Therefore, understanding the mechanism underlying the effect of light and photoperiod on PC cells can lead to finding novel non-invasive treatment to PC patients. In our study, environmental conditions were imposed on existing PC tumour. Another step in this research should be focused on the prevention of this kind of tumour. Therefore, it will be crucial to study the effects of LAN on PC in respect to light intensity, wave length as well as duration of exposure and to compare them with the epigenetic status of PC-related genes.

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