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INFLUENCE OF VARIOUS LIGHT REGIMES ON SOME CIRCADIAN RHYTHMS OF TRANSPLANTABLE MELANOMA B16

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BACKGROUND: Today it is known that disturbance of the lighting regime, expressed both by lengthening of the light period and its reduction, can not only affect the regulation of circadian rhythms of the organism, but also contributes to the initiation of neoplasm growth.

AIM: The aim of the study was to investigate circadian rhythmicity of melatonin level, some micromorphometric indices of tumor cells and expression of genes *Bmal1*, *Clock* and *Per2* in them in mice with transplanted melanoma B16.

MATERIALS AND METHODS: The study was conducted on 75 mice with subcutaneously transplanted melanoma B16, divided into 3 groups: control group, in which animals were kept under fixed light regime (light/darkness 10/14 hours with light on at 8:00 and off at 18:00), group under dark deprivation conditions, with animals kept under constant light 24 hours a day and group, in which animals were kept in constant darkness. The duration of the experiment was 2 weeks.

RESULTS: It was shown that under conditions of fixed light there are reliable circadian rhythms for all studied parameters, except for the nuclear-cytoplasmic ratio, the circadian rhythms of which was not revealed in any group. Constant darkness leads to rearrangement of all identified rhythms, and constant light causes destruction of all circadian rhythms except the Clock expression rhythm.

CONCLUSIONS: This study shows that light disturbances, whether constant light or constant darkness, lead to significant changes in the structure of the studied circadian rhythms.

Keywords: circadian rhythm; light pollution; melanoma; melatonin; clock genes.

ВЛИЯНИЕ РАЗЛИЧНЫХ СВЕТОВЫХ РЕЖИМОВ НА НЕКОТОРЫЕ ЦИРКАДНЫЕ РИТМЫ ТРАНСПЛАНТИРУЕМОЙ МЕЛАНОМЫ B16

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Обоснование. На сегодняшний день известно, что нарушение режима освещения, выражающееся как удлинением светового периода, так и его сокращением, не только может повлиять на регуляцию циркадных ритмов организма, но и способствует инициированию роста новообразований.

Цель — изучение циркадной ритмичности уровня мелатонина, некоторых микроморфометрических показателей клеток опухоли и экспрессии в них генов *Bmal1*, *Clock* и *Per2* у мышей с трансплантированной меланомой B16.

Abbreviations

CRs, circadian rhythms; NCR, nuclear-cytoplasmic ratio.

Материалы и методы. Исследование проведено на 75 мышах с трансплантируемой подкожно меланомой B16, разделенных на 3 группы: контрольную, в которой животных содержали при фиксированном световом режиме (свет/темнота 10/14 ч с включением света в 8:00 и выключением в 18:00); группу, находящуюся в условиях темновой депривации, с содержанием животных при постоянном освещении 24 ч/сут; группу, в которой животные пребывали в постоянной темноте. Длительность эксперимента составляла 2 нед.

Результаты. Показано, что в условиях фиксированного освещения достоверные циркадные ритмы присутствуют у всех изученных показателей, за исключением ядерно-цитоплазматического отношения, циркадных ритмов которого не выявлено ни в одной группе. Постоянная темнота приводит к перестройке всех выявленных ритмов, а постоянный свет вызывает разрушение всех циркадных ритмов, кроме ритма экспрессии *Clock*.

Заключение. Проведенное исследование показало, что нарушения режима освещения, будь то постоянное освещение или постоянная темнота, приводят к значительным изменениям в структуре изученных циркадных ритмов.

Ключевые слова: циркадный ритм; световое загрязнение; меланома; мелатонин; часовые гены.

Background

The circadian system of mammals is a complex mechanism that regulates many cyclic physiological processes by controlling gene expression and various metabolic pathways. Circadian rhythms (CRs) of functions and processes in the body, which are characterized by different amplitude and phase characteristics, are normally strictly synchronized with each other and with environmental factors. Such coordination provides the necessary order during these processes and allows maintaining the optimal level of functioning of the body systems [1, 2].

There is an association between the disruption of normal CR and the initiation of malignant tumor growth [3, 4].

The rhythmicity of cells in various mammalian organs is maintained by fluctuations in clock gene expression. However, numerous tumor suppressor genes and genes regulating the cell cycle and apoptosis are under the control of the circadian clock [5]. Zhou et al. [6] demonstrated that the CRs of multiple screening biomarkers can act as a prognostic factor in estimating overall survival in breast cancer.

The mouse epidermis is also susceptible to DNA damage induced by ultraviolet B-type radiation at night [7, 8]. The application of this radiation at night leads to more pronounced carcinogenesis in the skin than daytime application [9]. These diurnal differences do not occur in mutations in the core clock genes *Bmal1* and *Cry1/Cry2*, suggesting that circadian clocks regulate variability in sensitivity. The mechanisms underlying this phenomenon may be related to the low efficiency of DNA repair at night owing to the hourly periodicity of DNA repair variability. Alternatively, it may be a consequence of the high proportion of epidermal stem cells in the S-phase at night [10].

The daily rhythmicity of gene expression and the functioning of the "cell clock" in tumor cells are disrupted in neoplasms [11, 12].

The primary factor contributing to the disruption of biorhythms in the modern world is the imbalance between the duration of light and dark periods in an individual's daily cycle. A specific example of this imbalance is that caused by light pollution, which refers to exposure to light at night [13]. This environmental phenomenon is almost inevitable for a modern city dweller because of such social factors such as a high degree of digitalization of all labor and everyday processes, significant intensity of street lighting, shift or overtime work, and frequent transmeridian flights provoking desynchronosis when changing time zones [14]. The hypothesis of "circadian disruption," which is held by most chronobiologists, posits that exposure to light at night leads to a disruption of endogenous circadian rhythmicity and suppresses nocturnal secretion of melatonin by the pineal gland [15].

Darkness deprivation at night is associated with spontaneous oncogenesis in some mammals [16, 17]. Constant illumination along with other mechanisms can activate the development of chemically induced oncogenesis in model animals and promote the growth of transplantable tumors [18, 19].

In our previous studies, we found that prolonged exposure to constant illumination increases proliferation of melanoma cells, growth and spread of the tumor, development of more pronounced secondary changes, perivascular growth, and perineural invasion. In addition, keeping animals in constant darkness for long time results in a significant decrease in the intensity of tumor cell proliferation, accompanied by significant tumor regression in the absence of signs of lymphatic, intravascular, and intraneural invasion. The morphological characteristics of the tumor were confirmed by studying its cells. Our previous studies demonstrated a significant suppression of clock gene expression in melanoma cells under constant illumination, whereas under constant darkness, the opposite was observed [20, 21].

The bipartite aim of this study was to identify and examine the diurnal fluctuations and circadian rhythmicity of blood melatonin levels and to express *Per2, Bmal1*, and *Clock* genes in tumor cells to facilitate selection of micromorphometric indices of these cells in mice with transplantable melanoma B16.

Materials and methods

Male mice of the hybrid line BDF1 at the age of 8 weeks ($n = 75$), with body weights ranging from 21 to 22 g, were used. The animals were kept in the vivarium, and all experimental procedures were conducted in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, March 18, 1986). The series of studies to which this experiment belongs was approved by the A.P. Avtsyn Research Institute of Human Morphology.

Three equal groups of mice were used in the study.

The control group ($n = 25$) was kept under a fixed alternation of light and darkness using artificial illumination with fluorescent lamps (10/14 h). The lights were activated at 8:00 and deactivated at 18:00.

The experimental group I ($n = 25$) was kept under constant light conditions.

The experimental group II ($n = 25$) was kept in a constant state of darkness.

B16/F10 melanoma cell culture samples were injected into each animal according to the standard procedure [22]. The animals were slaughtered by cervical vertebra dislocation on day 15 after melanoma injection. The animals were removed from the experiment by the time-slice method four times a day (at 9:00, 15:00, 21:00, and 03:00) in groups of six or seven animals at each time, after which evisceration was performed.

The specimens were fixed in 10% neutral buffered formalin and processed by standard histological methods, with the final step being the pouring of the specimens into the Histomix histological medium (BioVitrum, St. Petersburg, Russia) and preparation of serial sections 5–6 μm thick on a Leica SM2010 R microtome (Wetzlar, Germany). The sections were stained with hematoxylin and eosin according to standard histological techniques [23]. The stained sections were subsequently fixed in BioMount permanent casting medium (BioVitrum, Russia).

A Leica DM 2500 microscope with a Leica DFC 290 digital camera (Germany) was used for the microscopic examination of the melanoma preparations. Karyotyping and cytometry were performed on 10 digital images of randomly selected fields of view obtained from each preparation under study at magnifications of ×200, ×400, and ×1000. The Fiji software package (a program based on ImageJ v2) was used with the requisite plugins to perform morphometric analysis [24]. Measurements were taken in micrometers after calibration of the object-micrometer scale. The nuclear-cytoplasmic ratio (NCR) of melanoma cells was determined by the formula: $NCR = S_n/S_{cell}$, where S_n is the area of the cell nucleus and Scell is the area of the cytoplasm [25].

For immunohistochemical analyses, polyclonal rabbit antibodies against *Per2, Bmal1*, and *Clock* (Cloud-Clone Corp., Houston, TX, USA) were used at a dilution of 1:200.

The processed slices were dehydrated in alcohols and xylene according to the standard scheme before they were fixed in BioMount permanent casting medium (BioVitrum, Russia).

Gene expression was evaluated as a percentage by counting stained cells and expressing their number as a percentage of the total number of cells on each slide (0%–100%) [26].

Blood samples were collected from mice four times a day at 9:00, 15:00, 21:00, and 03:00. The melatonin content in serum was quantified using a commercial enzyme immunoassay kit (USCN Life Science Inc., Wuhan, China) on a Sunrise analyzer (Sunrise-Basic-Tecan, Grödig, Austria).

The dynamics of the studied parameters over the course of a day were presented graphically in GraphPad Prism v8.41 (USA) and statistically analyzed. The D'Agostino-Pearson test was used to test for conformity to the normal distribution. If the data conformed to the normal distribution, Student's *t*-test for pairwise comparison and Tukey's test for comparison of three or more groups were used. Significant differences between the study groups and the control group were tested using the Dunnett's test. If the data deviated from the normal distribution, the Mann–Whitney U test for pairwise comparison and the Dunn test for comparison of three or more groups were used. The significance level was set at 5% ($p < 0.05$). In the plotting graphs, the arithmetic mean and standard deviation were displayed. The data are presented as mean ± standard deviation.

To calculate the amplitude and acrophase of CRs, we used the cosinor analysis method, which is an internationally recognized approach to the unified study of biological rhythms. This was conducted using the CosinorEllipse2006-1.1 program.

Cosinor analysis was designed to analyze wave processes and processing chronobiological data. During the analysis, the experimental data were approximated using the least squares method with a sinusoid. It was determined whether a reliable CR was present, and its acrophase and amplitude [27]. The data obtained from four measurements within 1 day were used for analysis.

Results

In animals in the control group, the maximum concentration of melatonin in blood was recorded at night, before it decreased to a minimum in the morning. Under conditions of constant darkness, the diurnal dynamics were less pronounced. In mice kept under constant illumination, melatonin was

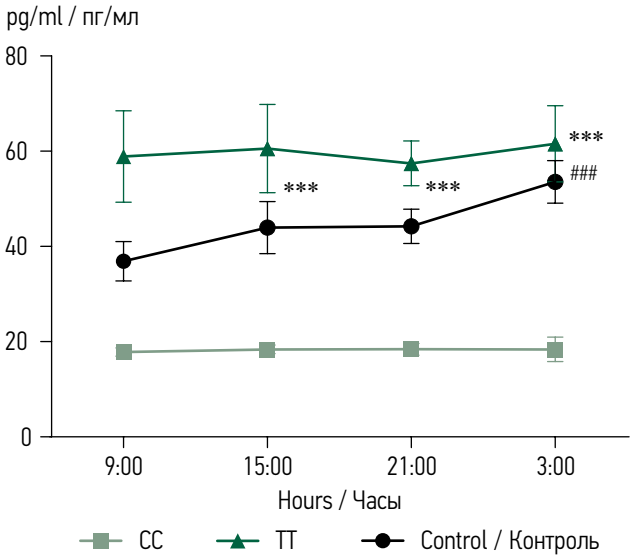

Fig. 1. Daily dynamics of melatonin content in the blood of mice. $^{***}p \leqslant 0{,}0005$ in comparison with the indicators at 9:00; $^{\#\#\#}p \leqslant 0{,}0005$ in comparison with the indicators at the previous time point. Here and further: CC — I experimental group; TT — II experimental group; Control — control group

Рис. 1. Суточная динамика содержания мелатонина в крови мышей. $^{***}p \leqslant 0{,}0005$ в сравнении с показателями в 9:00; $^{\#\#\#}p \leqslant 0{,}0005$ в сравнении с показателями в предыдущую временную точку. Здесь и далее: CC — I опытная группа; TT — II опытная группа; Контроль — животные контрольной группы

not detectable (Fig. 1). The cosinor analysis results corroborate this pattern, demonstrating that animals maintained under a fixed light regimen and subjected to constant darkness exhibited consistent CRs of melatonin content in their blood. These rhythms were characterized by an acrophase at 22:14 with an amplitude of 8.35 pg/mL in the first case and an acrophase at 05:16 with an amplitude of 4.25 pg/mL in the second case (Fig. 2).

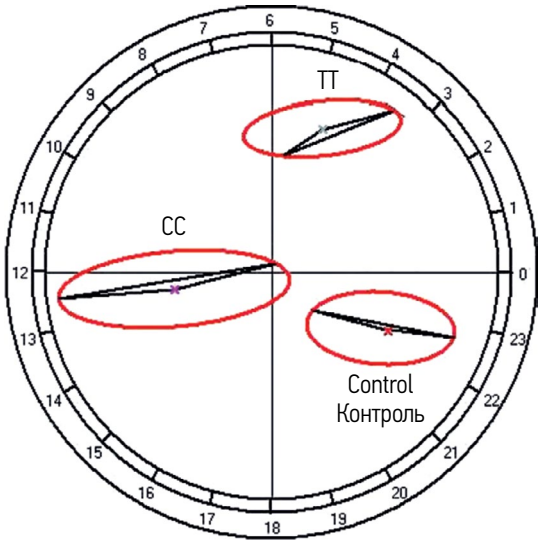

Fig. 2. Results of cosinor analysis of the daily dynamics of melatonin content in the blood of mice

Рис. 2. Результаты косинор-анализа суточной динамики содержания мелатонина в крови мышей

Analysis of the daily dynamics of micromorphometric parameters of melanoma cells revealed that the nuclei of melanoma cells in animals in the control group reached their maximum size at 21:00 and their minimum size at 15:00. In mice maintained in constant darkness, the same diurnal dynamic pattern was observed. However, in animals under constant illumination, the maximum size was recorded at 9:00 and the minimum at 21:00 (Fig. 3, *a*). The cosinor analysis results indicated that reliable CRs of cell nuclei size occurred only in the control group, with an acrophase at 15:48 with an amplitude of 1.93 μm^2, and in experimental group II, with an acrophase at 17:39 with an amplitude of 1.03 μm^2.

In contrast, in the control group, the daily dynamics of melanoma cell size showed a maximum at 15:00 and a minimum at 21:00. Under conditions of constant darkness, the maximum shifts to 9:00 and the minimum shifts to 3:00. Under constant light with the same maximum, the minimum cell size was recorded at 21:00 (Fig. 3, *b*). Cosinor analysis revealed that reliable CRs occurred only in the first two groups, with acrophases at 10:19 and 5:38 am with amplitudes of 29.66 and 12.24 μm^2, respectively.

The daily dynamics of NCR in the control and experimental (light/light) groups were characterized by a maximum at 21:00 and a minimum at 9:00 (Fig. 3, *c*). In contrast, in experimental group I, the maximum shifted to 9:00 and the minimum to 3:00. However, no significant CRs were recorded in either group as a result of cosinor analysis (Fig. 4).

The daily dynamics of *Bmal1* expression in tumor cells of control animals and mice kept in constant darkness exhibited a maximum at 9:00 and a minimum at 21:00. In contrast, animals kept under a constant light regime exhibited a minimum at 9:00 and a maximum at 3:00 (Fig. 5, a). The acrophases were recorded at 8:38 in the control group and at 2:26 in experimental group II, with amplitudes of 1.73% and 0.74%, respectively. In contrast, no significant CRs were recorded in experimental group I.

Clock expression in the control group was characterized by a maximum at 9:00 and a minimum at 21:00. In animals in experimental group I, the maximum was recorded at 3:00 and the minimum at 15:00. In mice of experimental group II, the maximum expression was observed at 3:00 and the minimum at 15:00 (Fig. 5, *b*). Cosinor analysis indicated the presence of CRs in all groups, with acrophases at 9:09, 0:31, and 0:24 and amplitudes of 1.54%, 1.50%, and 5.41%, respectively.

The expression of *Per2* in the control group was maximal at 3:00, before decreasing to a minimum at 15:00. In experimental group II, with a maximum at 9:00, *Per2* was least intensively expressed

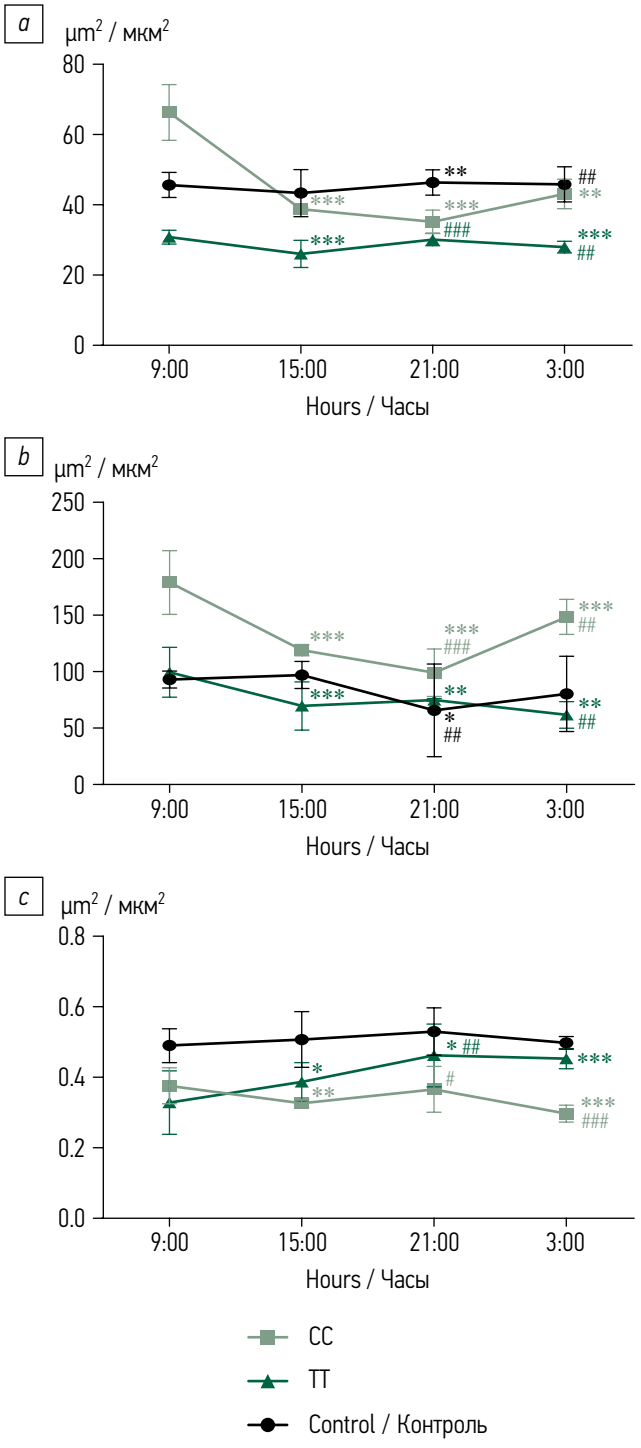

Fig. 3. Daily dynamics of the studied micromorphometric parameters of B16 melanoma cells: *a* — nuclei area; *b* — cell area; *c* — nuclear-cytoplasmic ratio. $*p \leqslant 0{,}05$, $**p \leqslant 0{,}005$, $***p \leqslant 0{,}0005$ in comparison with the indicators at 9:00; $^{\#}p \leqslant 0{,}05$, $^{\#\#}p \leqslant 0{,}005$, $^{\#\#\#}p \leqslant 0{,}0005$ in comparison with the indicators at the previous time poin

Рис. 3. Суточная динамика изучаемых микроморфометрических показателей клеток меланомы B16: *a* — площадь ядер; *b* — площадь клеток; *c* — ядерно-цитоплазматическое отношение. $*p \leqslant 0{,}05$, $**p \leqslant 0{,}005$, $***p \leqslant 0{,}0005$ в сравнении с показателями в 9:00; $^{\#}p \leqslant 0{,}05$, $^{\#\#}p \leqslant 0{,}005$, $^{\#\#\#}p \leqslant 0{,}0005$ в сравнении с показателями в предыдущую временную точку

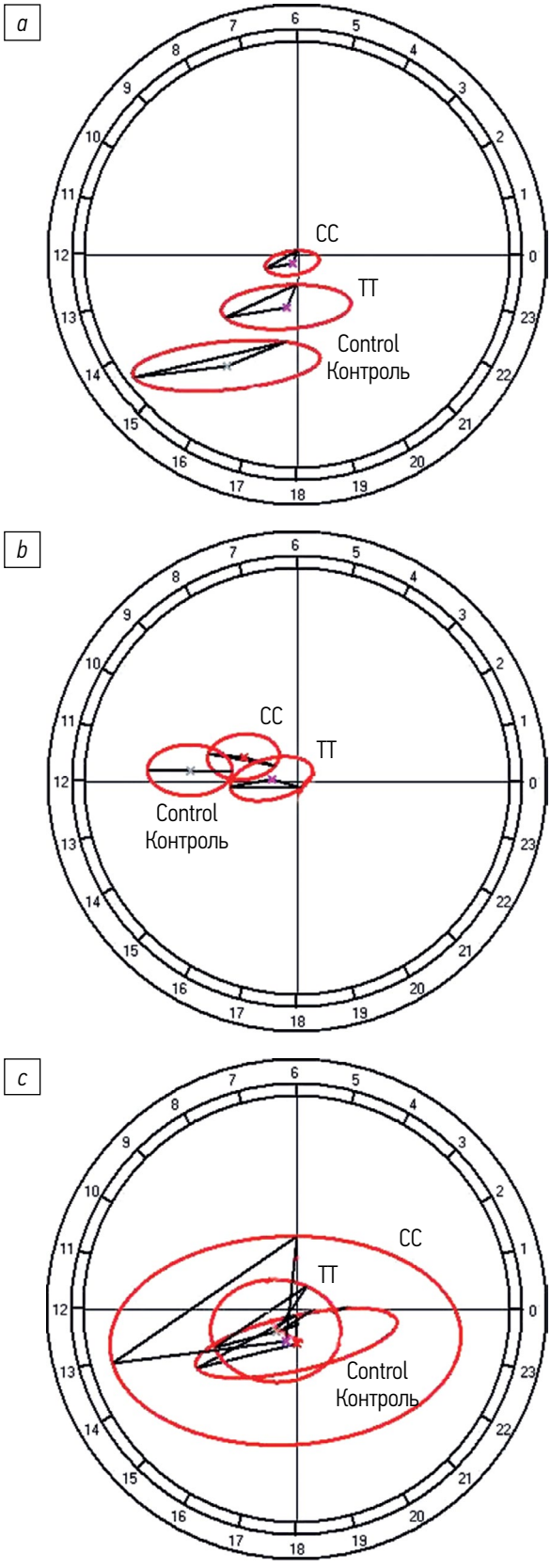

Fig. 4. Results of cosinor analysis of the daily dynamics of the area of the nucleus (*a*), cell (*b*) and nuclear-cytoplasmic ratio (*c*) of B16 melanoma cells

Рис. 4. Результаты косинор-анализа суточной динамики площади ядра (*a*), клетки (*b*) и ядерно-цитоплазматическое отношение (*c*) клеток меланомы B16

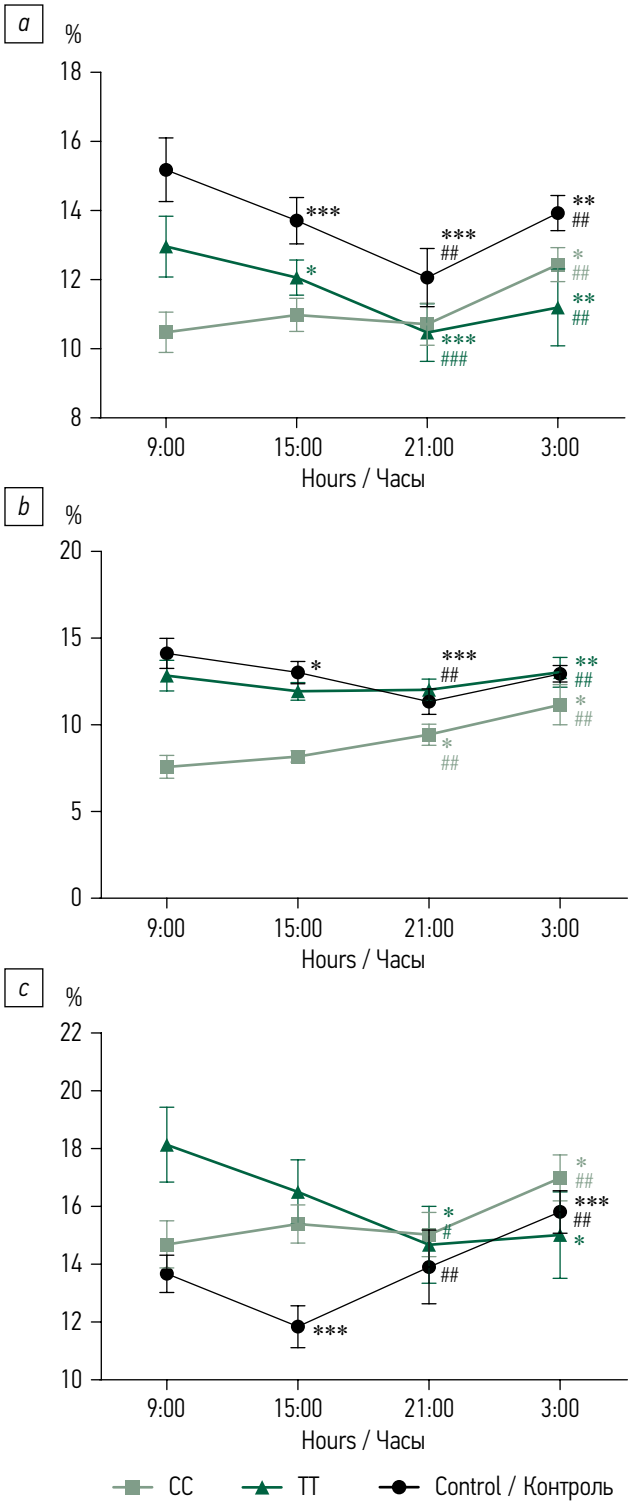

Fig. 5. Daily dynamics of expression of the studied genes in B16 melanoma cells: *a* — *Bmal1*; *b* — *Clock*; *c* — *Per2*. $^{*}p \leqslant 0{,}05$, $^{**}p \leqslant 0{,}005$, $^{***}p \leqslant 0{,}0005$ in comparison with the indicators at 9:00; $^{\#}p \leqslant 0{,}05$, $^{\#\#}p \leqslant 0{,}005$, $^{\#\#\#}p \leqslant 0{,}0005$ in comparison with the indicators at the previous time point

Рис. 5. Суточная динамика экспрессии изученных генов в клетках меланомы B16: *a* — *Bmal1*; *b* — *Clock*; *c* — *Per2*. $^{*}p \leqslant 0{,}05$, $^{**}p \leqslant 0{,}005$, $^{***}p \leqslant 0{,}0005$ в сравнении с показателями в 9:00; $^{\#}p \leqslant 0{,}05$, $^{\#\#}p \leqslant 0{,}005$, $^{\#\#\#}p \leqslant 0{,}0005$ в сравнении с показателями в предыдущую временную точку

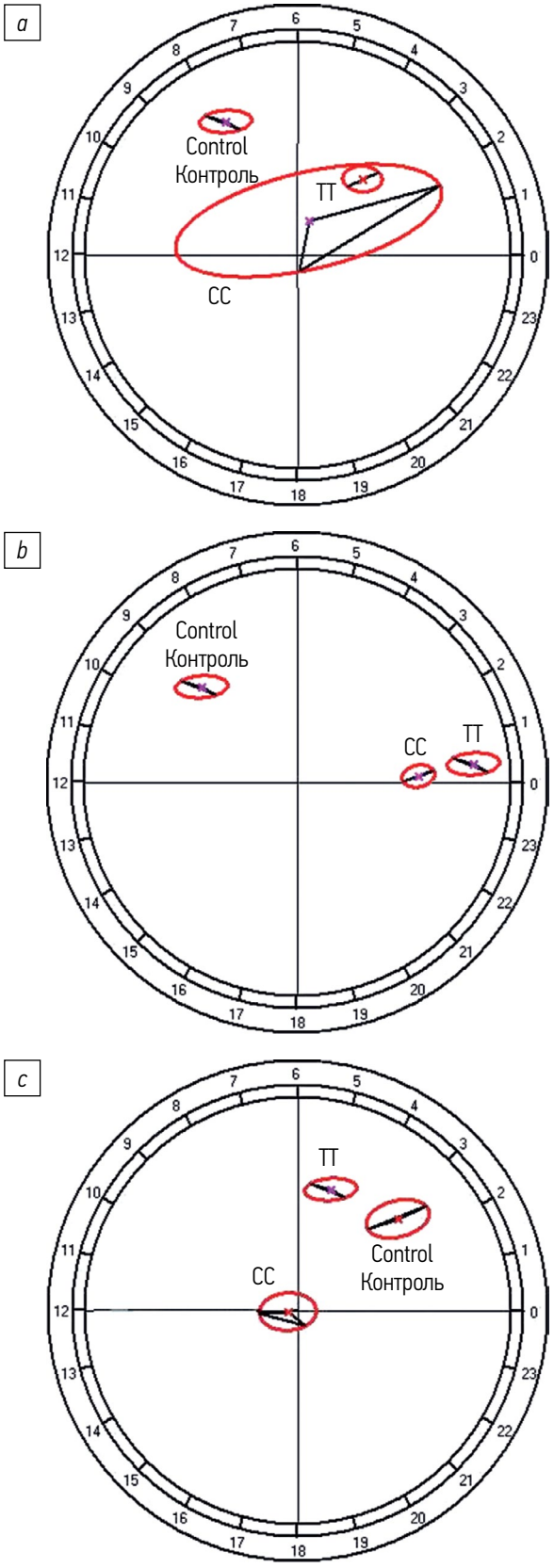

Fig. 6. Results of cosinor analysis of the daily dynamics of expression of *Bmal1* (*a*), *Clock* (*b*) and *Per2* (*c*) in B16 melanoma cells

Рис. 6. Результаты косинор-анализа суточной динамики экспрессии *Bmal1* (*a*), *Clock* (*b*) и *Per2* (*c*) клеток меланомы B16

at 21:00. In animals kept under constant light, the maximum expression was observed at 3:00 and the minimum at 9:00 (Fig. 5, *c*). A notable change in this parameter was observed only in the control group and experimental group II. In the control group, the amplitude of the rhythm was 1.96% with acrophase at 2:41, whereas in experimental group II, the amplitude was 0.82% with acrophase at 5:10 (Fig. 6).

Discussion and conclusions

Our results showed that light disturbances, whether constant illumination or constant darkness, led to significant changes in the structure of the studied CRs.

Our findings indicate that the melatonin CR characteristic of a fixed light regime under conditions of constant darkness is disrupted by constant illumination. This phenomenon can be explained by the fact that melatonin is synthesized and secreted by the pineal gland at night under a normal light/dark ratio. The endogenous secretion rhythm of this hormone is generated by the suprachiasmatic nuclei of the hypothalamus, which strictly obeys the light cycle. Light signals can suppress melatonin production or synchronize it according to the illumination schedule (photoreduction of rhythm) [28]. Consequently, the normal light/dark cycle is disrupted, and the circadian rhythmicity of melatonin production is disrupted. Under conditions of constant darkness, the rhythm becomes free and unaffected by external time sensors, whereas under constant illumination, melatonin production by the pineal gland is significantly reduced.

The effects of melatonin on cells can be exerted through melatonin receptors on the cytoplasmic membrane. Three types of M receptors occur in the plasma membrane: MT1 (M1a, MTNR1A), MT2 (M1b, MTNR1B), and MT3 (M1c, MTNR1C) [29]. The first two types also occur in humans. They belong to the serpentine receptor class and are associated with G-proteins. MT1 receptors have been detected in the suprachiasmatic nuclei of the hypothalamus, anterior lobe of the pituitary gland, intestine, kidneys, and melanoma cells [30].

The expression rhythms of *Bmal1* and *Per2* in tumor cells were preserved under constant darkness but disrupted under constant illumination. This can be attributed to changes in melatonin production and the CR response, which arise under the influence of constant darkness, destruction of CR, and deficiency in epiphyseal melatonin under constant illumination. One of the physiological effects of melatonin is the synchronization of the expression of clock genes, primarily *Bmal1* and *Per2*. The absence of disruption to the rhythm of the *Clock* expression under constant illumination and constant darkness raises several issues. Notably, the acrophases of this rhythm in the groups with light disturbance practically coincide. Many studies devoted to the study of melatonin participation in the work of the main genes of the biological clock at various stages of their expression in tissues have yielded data that cannot be easily fitted into a simple model. The spectrum of potential effects of melatonin on the regulation of the main genes of the biological clock is vast, hierarchical, and likely specific to various tissues and organs [31, 32].

Analysis of the rhythmicity of micromorphometric indices also indicates that reliable CRs of tumor cell areas and their nuclei occur only in the first two groups (control and dark/dark) but collapse under constant illumination. In contrast, the CR of NCR was not observed in any group, perhaps because of the high frequency of morphofunctional changes in the tumor cells.

The effect of melatonin on morphological and functional parameters can occur through the MT1-, MT2-, and possibly MT3-melatonin receptors of the cell, as well as in the absence of these receptors. This is because melatonin, which is lipophilic and has a small molecular size, can penetrate both the cytoplasm and karyoplasm without the help of receptors. However, this issue requires further study.

Our findings indicate that light disturbance induces substantial alterations in the CRs of transplantable melanoma B16 cells. In addition, constant darkness typically induces structural changes in the studied rhythms, whereas constant illumination results in their destruction.

Additional information

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Ethics approval. This research was approved by the Bioethical Committee of the Avtsyn Research Institute of Human Morphology (protocol No. 34 (10) dated 14.03.2022).

Competing interests. The authors declare no competing of interest.

Author contribution. All authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study.

Personal contribution of each author: *D.A. Areshidze, M.A. Kozlova, Z.V. Gioeva, D.V. Mishchenko, T.V. Bezuglova, U.Yu. Allayarova* — concept and design research; *M.A. Kozlova, A.I. Anurkina* — staging experiments, processing material; *D.A. Areshidze,*

M.A. Kozlova, V.P. Chernikov, M.V. Mnikhovich — conducting analysis and interpretation of the obtained data; *D.A. Areshidze, M.A. Kozlova, M.V. Mnikhovich* — writing the text of the article; *D.A. Areshidze, M.A. Kozlova, V.P. Chernikov* — editing the text of the article.

Дополнительная информация

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Вклад авторов. Все авторы внесли существенный вклад в разработку концепции, проведение исследования и подготовку статьи, прочли и одобрили финальную версию перед публикацией.

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