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Melatonin modulates the action of near infrared radiation on cell adhesion

Abstract: The adhesion of human cervical cancer (HeLa) cells to a glass matrix is evaluated following their irradiation in a suspension with a pulsed near-infrared (IR) light-emitting diode (wavelength 820 nm, pulse repetition frequency 10 Hz, irradiation dose 16–120 J/m^2) when melatonin (4×10^{-11} to 4×10^{-5} M) is added to cell suspension immediately before or after the irradiation. Also, the dependence of visible-to-near-IR radiation (600–840 nm, 52 J/m^2) on cell adhesion (action spectrum) is recorded in absence and presence of melatonin (4×10^{-6} M). It is found that melatonin in pharmacological concentrations (but not in physiological range) inhibited cell adherence. Irradiation of cells before or after melatonin treatment normalizes cell adhesion to control level. Melatonin in pharmacological concentrations eliminates stimulation of cell attachment induced by irradiation. Pre-treatment (but not post-treatment) with melatonin in the physiological concentration eliminates cell adhesion stimulation induced by irradiation. Melatonin modifies the light action spectrum significantly in near IR region (760–840 nm only). Thus, the peak at 820–830 nm characteristic for the light action spectrum is fully reduced.

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Introduction

Monochromatic visible-to-near-infrared (IR) radiation from lasers and diodes is used to stimulate wound healing and tissue regeneration; also, it is used for treatment of pain [1]. Experimental evidence was presented that there exists a relationship between pain attenuation by light at 632.8 [2] and 820 nm [3] and serotonergic mechanisms. Recent developments indicate that melatonin may have analgesic effects in humans [4].

Melatonin is the major messenger of light-dependent periodicity in humans. Light-emitting diodes can be used to phase-delay the melatonin rhythm [5, 6]. Melatonin is a significant scavenger of free radicals and antioxidant at both physiological and pharmacological concentrations [7]. The antioxidative mechanism of melatonin is different from the action of antioxidants such as vitamins C and E or glutathione. It does not undergo redox cycling and therefore it does not cause oxidation [8].

An investigation into cellular and molecular effects of monochromatic visible-to-near-IR radiation demonstrated that this type of radiation stimulates various metabolic responses in cells including DNA synthesis and cell adhesion [9]. The molecular mechanism underlying the effect of increase of cell adhesion induced by monochromatic visible-to-near-IR radiation has not as yet been established. It has been assumed that the action of irradiation on cell attachment is the result of its absorption by mitochondria, followed by the transduction of photosignal from the mitochondria to the cell membrane via rapid changes in cell homeostatic parameters [10].

Melatonin in both pharmacological (10^{-5} M) and physiological (10^{-9} M) concentrations was found to eliminate increased HeLa cell adhesion induced by radiation with 820 nm [11]. In these pilot experiments, melatonin was used as one of several antioxidants.

The aim of the present work is to study cell adhesion as influenced by melatonin concentration (in broad range from 10^{-11} to 10^{-5} M), light wavelength, and treatment sequence. The studies on the simultaneous action of melatonin and various wavelengths of light may help to answer the question, in which manner melatonin interacts with cells.

Materials and methods

Cell culture

The HeLa cells, initially obtained from the Institute of Virology (Moscow, Russia) were grown in closed scintillation vials in 2 ml of medium 199, with 10% bovine serum and 100 U/ml of penicillin and streptomycin added. The experiments were performed within 72 h of plating, when the culture was in the exponential growth phase. The monolayer was harvested using 0.02% Versene solution (37°C), and the suspension was prepared in medium 199 containing 10% of bovine serum. Cellular suspension samples were irradiated in special glass cuvettes (volume 130 μl, layer thickness 4 mm, number of cells 85,000) as

described in detail elsewhere [10]. Cell culture processing was performed in dark or under dim natural light. Extraneous illumination (sunshine or artificial light) was avoided.

Light source and irradiation

A GaAlAs light-emitting diode (Polyus; Moscow, Russia) was used for irradiation. The technical characteristics of this light source were as follows: wavelength $\lambda = 820 \pm 10$ nm, pulse repetition frequency 10 Hz, pulse width 20 ms, and duty factor (pulse duration-to-pulse period ratio), 20%. Irradiation was performed through an optical fibre, so that the exposed suspension surface (0.38 cm^2) was totally covered by a homogeneous light spot. The radiation intensity at the suspension level was 3.54 W/m^2, and the radiation dose varied from 8 to 120 J/m^2 (exposure time, from 10 to 170 s). Intensity measurements were taken using a Spectra Physics M404 power meter. Irradiation was performed at room temperature in the dark.

Monochromatic radiation from 600 to 840 nm was obtained by means of a monochromator designed by Dr A. Lifshits at the Institute of Spectroscopy, Russian Academy of Sciences and described elsewhere [10]. Action spectra were recorded in the same special glass cuvettes described above. Ten microlitres of a melatonin solution was added to each sample cell immediately before the irradiation. Light intensity was 1.3 W/m^2, dose 52 J/m^2, irradiation time 40 s. These parameters were found to be optimal in previous experiments [10].

Adhesion assay

The criterion by which changes in the adhesion properties of the cell membrane were judged was the number of cells that attached themselves to the bottom of the cuvette in the course of 30 min at 37°C. This time was found earlier to be optimal for cell attachment in our experimental conditions [10]. The optimal experimental conditions (number of cells per vial, incubation time) were established in a special series of experiments such that the stimulatory and inhibitory effects of the chemicals could be measured under the same conditions. After incubation, the nutrient medium was removed, and the cuvettes were washed with phosphate-buffered saline (PBS) to remove all non-attached cells. The attached cells were trypsinized, and their number was counted with a haemocytometer. Each result represented the mean of at least 10 independent measurements.

Chemicals

Melatonin was purchased from Sigma (St Louis, MO, USA) and stored under an argon atmosphere at −24°C. Fresh solutions were prepared before every experiment. Melatonin was first dissolved in a minimal volume of absolute ethanol and then transferred to PBS, so the concentration of ethanol in final solutions of melatonin was not higher than 1×10^{-6} M. Possible toxicity of melatonin solutions was checked by the trypan blue exclusion test; all concentrations used were found to be non-toxic in our experimental conditions (viability of cells >95%). Melatonin has no absorbance in the red-near-IR region used for the irradiation (checked spectrophotometrically).

Statistical analysis

The results obtained were statistically processed by means of the STATISTICA v. 4.5 and GRAPHPAD program packages and expressed in terms of the mean value ± S.D. (from 10 measurements), $P < 0.05$ was considered significant using the ANOVA and Student's *t*-tests.

Results

In our experimental conditions, 42.5 ± 2.5% of the total number of cells in a cuvette (85,000) attach themselves to the bottom of the vial. Irradiation increased the number of the cells attached to the glass in a dose-dependent manner. The dose-dependence of cell adhesion is presented in Fig. 1 (curve marked by *hv*). This curve is bell-shaped, with a maximum at 60 J/m^2; the percentage of the adherent cells at this point was 64.5 ± 3.1%.

In the following experiments, melatonin was added in three concentrations (4×10^{-5}, 4×10^{-9}, 4×10^{-11} M) immediately before or after the irradiation of samples in doses from 16 to 120 J/m^2. The treatment of cells with melatonin at a concentration of 4×10^{-5} (Fig. 1A) or 4×10^{-9} M (Fig. 1B) before or after the irradiation inhibited cell adhesion stimulation caused by irradiation (curves denoted as *hv* + Mel and Mel + *hv*). Melatonin in a concentration of 4×10^{-9} M had no statistically significant influence on cell adhesion (per cent of adherent cells 34.8 ± 7.0 as compared with control of 42.5 ± 2.5%). Melatonin in concentration 4×10^{-5} M inhibited cell adhesion (per cent of adherent cells, 26.5 ± 5.2, $P < 0.05$). This probably explains why in Fig. 1B the curves of dose dependencies (melatonin and irradiation in both sequences) are near the control level, but in Fig. 1A, these curves are at lower cell attachment levels than the control.

The situation is quite different when melatonin is used in a concentration of 4×10^{-11} M (Fig. 1C). Melatonin itself stimulated cell attachment (51.1 ± 5.3%, $P < 0.05$). Treatment of cells with melatonin after irradiation had no effect (curve *hv* + Mel) as compared with irradiated cells (curve *hv* in Fig. 1C). Application of melatonin before irradiation eliminated the stimulation of cell attachment when light doses were less than optimal (60 J/m^2). Increasing the light dose increased the per cent of attached cells in dose-dependent manner (curve Mel + *hv* in Fig. 1C).

In the next series of experiments, the radiation was used in one dose only (60 J/m^2) but the concentration of melatonin varied from 4×10^{-11} to 4×10^{-5} M. Fig. 2 presents the percentage of cell attachment on the concentration of melatonin without irradiation (curve 1 in Fig. 2A,B) and the same concentration dependencies when cell suspensions were irradiated at a dose of 60 J/m^2 before treatment with melatonin (curve 2 in Fig. 2A) or after the treatment (curve 3 in Fig. 2B).

Melatonin in concentration range from 4×10^{-10} to 4×10^{-7} M had no statistically significant influence on cell attachment; in concentration 4×10^{-11} M it stimulated cell attachment and at concentrations 4×10^{-6} and 4×10^{-5} M,

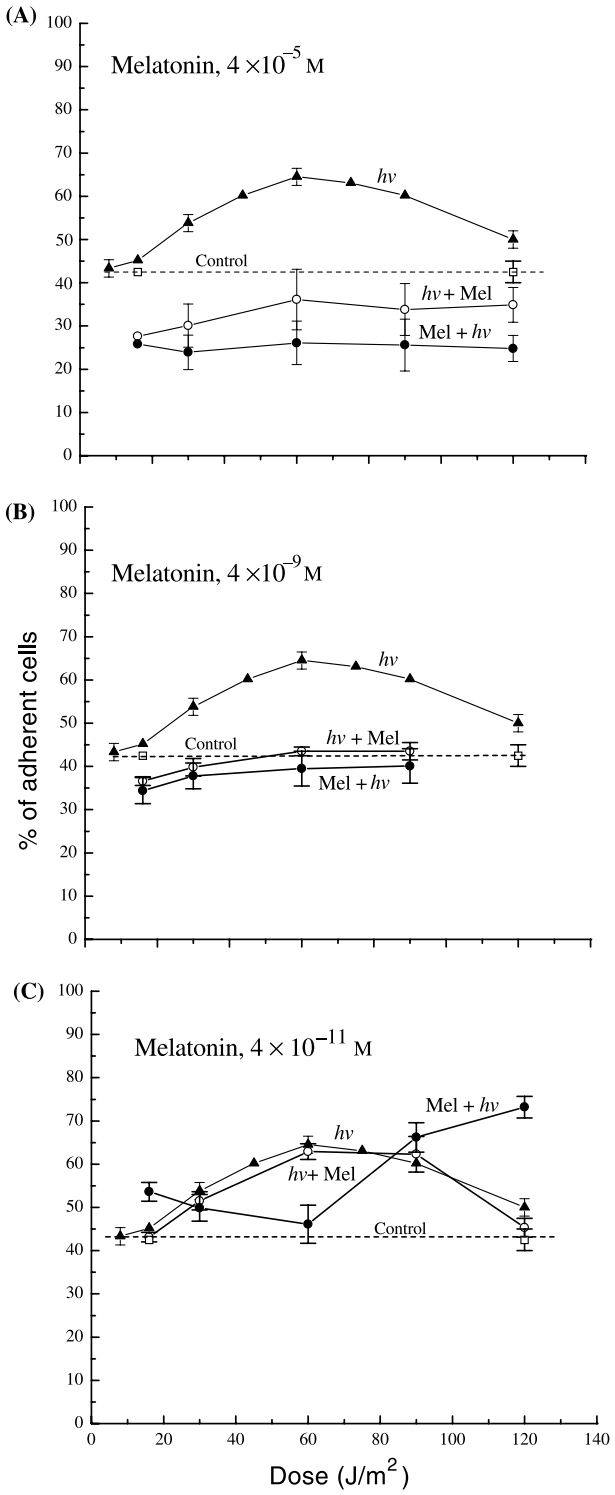

Fig. 1. Effects of melatonin at concentrations of: (A) 4×10^{-5} M, (B) 4×10^{-9} M, (C) 4×10^{-11} M on cell attachment depending on irradiation ($\lambda = 820$ nm) dose. Dashed lines show the attachment of control cells; the curves denoted with *hv* indicate the dose-dependence of light action without melatonin. Percentage of adherent cells in control experiments with melatonin is the following: 26.5 ± 5.2 (4×10^{-5} M), 34.8 ± 7.0 (4×10^{-9} M) and 51.1 ± 5.3 (4×10^{-11} M).

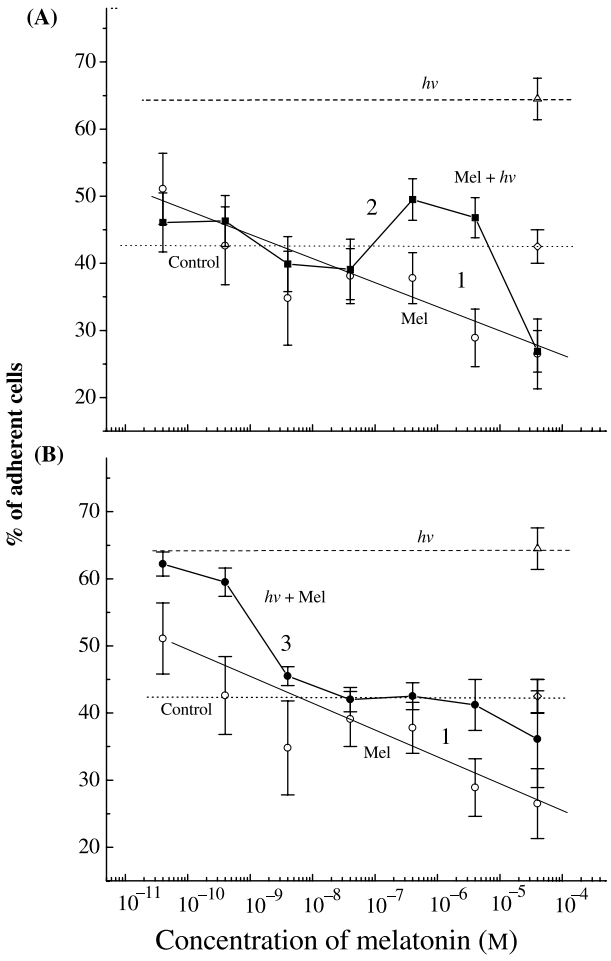

Fig. 2. Cell attachment to glass depending on concentration of melatonin. Melatonin was added (A) before (curve 2) and (B) after (curve 3) irradiation (dose 60 J/m^2, $\lambda = 820$ nm). Curve 1 denotes percent of adherent cells when melatonin is added to cell suspensions but cells are not irradiated. Dashed line denoted by *hv* shows the per cent of adherent cells under irradiation. Dotted line marks the attachment of control cells.

melatonin inhibited cell attachment in our experimental conditions (Fig. 2A,B, curve 1).

Irradiation influences cell attachment depending on melatonin concentrations. The final result (increase or decrease of cell attachment) also depends on the sequence of treatments. There were two ranges of melatonin concentration (from 4×10^{-11} to 4×10^{-9} M and from 4×10^{-8} to 4×10^{-5} M), which were characterized by different modes of action on cell attachment when applied together with radiation (Fig. 2A,B).

To clarify further this situation, a scheme is designed in Fig. 3 on the basis of the data from Fig. 2. Fig. 3 denotes three regions of different effects. In region I, light-induced stimulation of cell attachment is suppressed by melatonin back to the control level. This is characteristic for both physiological and pharmacological concentrations in sequence Mel + *hv* and for pharmacological concentrations of melatonin in sequence *hv* + Mel. Region II is characteristic for pharmacological concentrations of melatonin only.

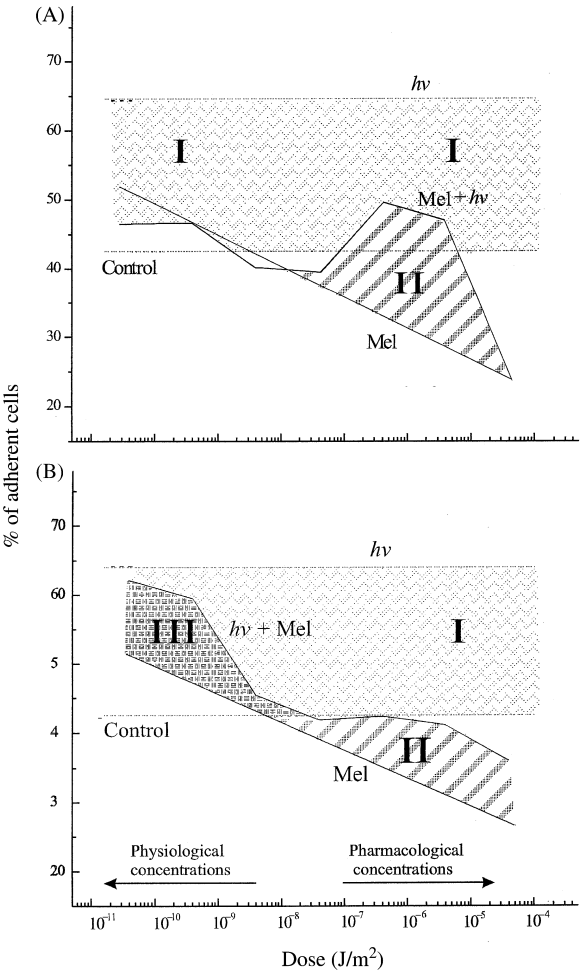

Fig. 3. Cell attachment to glass depending on concentration of melatonin. A scheme based on the data in Fig. 2. The marks are the same as in Fig. 2. An explanation of for regions I, II and III is provided in the text.

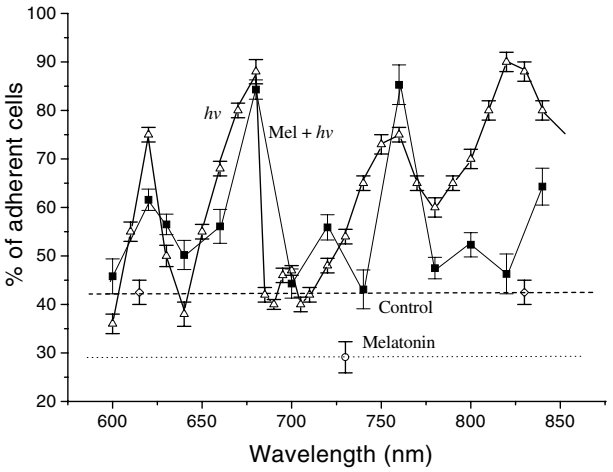

Fig. 4. Cell attachment to glass depends on wavelength used for irradiation of samples of a suspension without melatonin (curve marked by *hv*) or with melatonin (4×10^{-6} M, curve Mel + *hv*) added before irradiation (dose 52 J/m^2, intensity 1.3 W/m^2, irradiation 40 s, adhesion measurements performed 30 min after the irradiation). Dashed line indicates the attachment of the non-irradiated suspension (control). Dotted line indicates the attachment of the suspension in presence of melatonin (4×10^{-6} M) without irradiation.

In spite of the treatment sequence, the irradiation eliminated the suppression of cell attachment caused by melatonin and normalized it to the control level. Region III is characteristic for the sequence *hv* + Mel only. In this region, post-irradiational treatment of cells with low concentrations (4×10^{-11} to 4×10^{-10} M) of melatonin had little effect on cell adhesion stimulation induced by irradiation; by increasing the concentration of melatonin light-induced stimulation of cell adherence decreased.

Fig. 4 presents the dependence of the percentage of attached cells on wavelength used for irradiation (the action spectrum marked by *hv* in Fig. 4). Also, the same sort of dependence when cells were irradiated in presence of melatonin in concentration 4×10^{-6} M is presented (curve Mel + *hv* in Fig. 4). Cell attachment to the glass surface was stimulated upon irradiating the cellular suspension samples with light in the wavelength ranges of 600–625, 645–700 and 720–840 nm with maxima at 620, 680, 760 and 820–830 nm, respectively (curve denoted by *hv* in Fig. 4).

Melatonin modified this action spectrum significantly in the near IR region only (from 770 to 840 nm in our experiment). In this particular region, the peak at 820–830 nm, which is characteristic for a light action spectrum (curve *hv*), was absent in the spectrum recorded in the presence of melatonin (curve Mel + *hv* in Fig. 4). In other spectral regions, the peak at 620 nm was below and peak at 760 nm was above the respective points for the control curve. The peak positions were not altered in the presence of melatonin (curve Mel + *hv* in Fig. 4).

Discussion

The cell surface and its adhesive interactions are involved in regulation of such processes as cell growth, differentiation, morphogenesis, wound repair, formation of metastasis, etc. Both cell–cell adhesion and cell–matrix adhesion molecules, isolated and characterized thus far, are glycoproteins, proteoglycans, and glycolipids [12], i.e. the classes of molecules that do not absorb visible and near IR radiation. This means that the effect of radiation on cell adhesion is apparently not associated with the direct action of radiation on adhesion molecules.

The increase of HeLa cell adhesion to a glass substratum under irradiation has a well-structured action spectrum (the dependence of this parameter on the wavelength used for irradiation, Fig. 4). This circumstance indicates the existence of a photoacceptor as the action spectrum resembles the absorption spectrum of a primary photoacceptor [13]. The action spectrum presented in Fig. 4 and marked by *hv* is believed to reflect the absorption spectrum of a mixed valence form of cytochrome *c* oxidase located in mitochondria [9].

Results obtained in our experiments show that, first, both melatonin and irradiation influence cell attachment when applied separately, depending on their concentration and

dose, respectively. Secondly, it is evident that when applied together, melatonin and radiation modulate the action of each other.

It is possible that melatonin modulates the action of light in mitochondria, i.e. in the first steps of light action (photon absorption and primary photochemistry). The modulation of light action spectrum by melatonin (Fig. 4) supports this suggestion. If this is the case, the interaction could occur in the vicinity of Cu_A (this chromophore in oxidized state gives 85% of absorption in the peak with maximum at 820–830 nm [14]). As the peak is eliminated by melatonin (Fig. 4), one could suppose that Cu_A becomes more reduced in the presence of melatonin. It is known that the peak at 820–830 nm disappears by reduction of cytochrome *c* oxidase [14]. Melatonin, as a highly lipophilic small molecule, quickly reaches cellular organelles including mitochondria [15]. In principle, a direct action of melatonin to respiratory chain carriers including cytochrome *c* oxidase is possible [15].

Melatonin may regulate the mitochondrial redox state, as it was shown experimentally for brain and liver mitochondria [16]. Melatonin at a concentration of 1×10^{-7} M increased the activity of the complexes I and IV (cytochrome *c* oxidase) of the respiratory chain. In another set of experiments, melatonin was found to increase the activity of both complexes in the concentration of 1×10^{-9} M [15].

Melatonin may act on later reactions followed after light absorption and primary photochemistry, i.e. in cellular signalling reaction cascades. Melatonin can act during secondary reactions as a free-radical scavenger or, for example, through NF-κB or other transcription factors. The action through modulation of NF-κB levels has been discussed both for visible-to-near-IR radiation [9] and melatonin [17].

Conclusions

We have found in the present work, first, that melatonin in pharmacological concentration range (but not in physiological range) inhibits cell adherence. Secondly, irradiation of cells at $\lambda = 820$ nm before or after melatonin treatment in pharmacological concentrations normalizes cell adhesion to the control level. Melatonin in pharmacological concentrations eliminates stimulation of cell attachment induced by irradiation at 820 nm. The third finding is that in the physiological concentration range, only pre-treatment with melatonin eliminates cell adhesion induced by irradiation. Fourthly, melatonin modifies the light action spectrum in the range of 600–840 nm, most significantly in the near-IR region (760–840 nm).

Additional comment about possible medical/biological significance of results obtained

Is it possible to extrapolate the results obtained in vitro in the present work to in vivo situations? The following speculation can be provided. Typical night-time concentrations of melatonin in humans are near 10^{-9} M and daytime concentrations are near 10^{-11} M [15]. This suggests that cell adhesion would not be increased by the irradiation (laser therapy) in night-time, only during the daytime (Fig. 2A). Secondly, in the presence of pharmacological concentrations of melatonin (e.g. intake of this neurohormone or some drug, which increases the synthesis of melatonin), cell adhesion could be increased during laser therapy (Fig. 2A). Thirdly, comparing the dose-dependencies in Fig. 1A–C, it appears that melatonin influences cell adhesion much powerfully as compared with radiation at 820 nm. Only at the concentration of 4×10^{-11} M (Fig. 1C) the action of melatonin and radiation are of comparable strength.

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