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Melatonin exerts oncostatic capacity and decreases melanogenesis in human MNT-1 melanoma cells

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Abstract

Melanogenesis is a key parameter of differentiation in melanocytes and melanoma cells; therefore, search for factors regulating this pathway are strongly desired. Herein, we investigated the effects of melatonin, a ubiquitous physiological mediator that is found throughout animals and plants. In mammals, the pineal gland secretes this indoleamine into the blood circulation to exert an extensive repertoire of biological activities. Our in vitro assessment indicates an oncostatic capacity of melatonin in time-dependent manner (24, 48, 72 hours) in highly pigmented MNT-1 melanoma cells. The similar pattern of regulation regarding cell viability was observed in amelanotic Sk-Mel-28 cells. Subsequently, MNT-1 cells were tested for the first time for evaluation of melanin/melatonin interaction. Thus primary, electron paramagnetic resonance (EPR) spectroscopy demonstrated that melatonin reduced melanin content. Artificially induced disturbances of melanogenesis by selected inhibitors (N-phenylthiourea or kojic acid) were slightly antagonized by melatonin. Additionally, analysis using transmission electron microscopy has shown that melatonin, particularly at higher dose of 10^{-3} mol/L, triggered the appearance of premelanosomes (stage I-II of melanosome) and MNT-1 cells synthesize de novo endogenous melatonin shown by LC-MS. In conclusion, these studies show a melanogenic-like function of melatonin suggesting it as an advantageous agent for treatment of pigmentary disorders.

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AUTHOR CONTRIBUTION

KK conceived, designed the experiments, analyzed obtained data, and then wrote the manuscript. TKK conducted all experiments and then analyzed the LC-MS data. BB performed electron microscopy imaging and together with EP analyzed the results. MS and KM conducted electron paramagnetic resonance spectroscopy assessment and evaluated obtained data. AS and ATS supported the interpretation of results while ATS, RJR, KS, MB, KK drafted the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Keywords

electron paramagnetic resonance spectroscopy; liquid chromatography-mass spectroscopy; melanogenesis; melanoma cells; melatonin; transmission electron microscopy; tyrosinase activity

1 | INTRODUCTION

Melanogenesis is a multistage process involving melanin synthesis in melanosomes, the transport of melanosomes to the dendrite tips of the melanocytes, and their subsequent release. In melanocytes and in melanoma cells, melanin synthesis is controlled by a cascade of enzymatic reactions beginning with the oxidation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosinase (TYR), the rate-limiting enzyme of melanogenesis.[1,2] Among the hormonal regulators of melanin synthesis,[3] α-melanocyte-stimulating hormone (α-MSH),[4-8] a product of POMC processing, plays an important role. It interacts with a specific cell surface melanocortin 1 receptor (MC1R) stimulating melanin synthesis and other differentiated functions of melanocytes.[8,9] Furthermore, the cyclic adenosine monophosphate (cAMP) pathway plays a key role in the regulation of melanogenesis through up-regulation of the transcription microphthalmia-associated transcription factor (MITF) and subsequent melanogenic enzymes including TYR.[10-12] Thus, considering the enhanced number of individuals with pigment disorders, an agent with potent regulatory capacities of melanogenesis is of broad therapeutic interest.

Melatonin (N-acetyl-5-methoxytryptamine) is a methoxyindole hormone and a bioregulator which is present in almost all biological systems including animals, plants, and microbes.[13-17] It is synthesized in the pineal gland[18] but also in other cells[19-23] including in the human skin.[24-28] Melatonin is well-known to regulate circadian rhythm in humans. Additionally, it has many other effects including regulation of immune and endocrine functions, and it exhibits anti-apoptotic,[29-32] and anti-inflammatory[33] properties against the internal and environmental insults.[13,14,16,19,22,28,34-40] These responses are mediated either through binding to membrane-bound melatonin receptors (MT1 or MT2), receptor-independent mechanisms, or through activation of nuclear receptors.[13,14,16,22,41,42] Expression of membrane-bound cell surface MT receptors in the skin is variable, depending on the species. For instance, skin from the C57BL/6 mouse predominantly or exclusively expresses MT2.[43,44] Differently, human skin expresses both receptors,[17,27,45] although with a strong bias toward MT1 (the predominant form found in both whole skin and cultured cells).[26,46] We have recently shown that highly pigmented human MNT-1 melanoma cells possess membrane-bound melatonin receptors (MT1 or MT2) indicating high-affinity binding for melatonin.[31]

To date, extensive studies have been focused on melatonin's role in general regulation of body homeostasis.[13,14,16,22] It is known that locally produced melatonin plays a role in the regulation of skin physiology,[17,24,27,28,47] and exogenously applied melatonin exerts pro-differentiation effects in human skin.[28,48,49] Originally melatonin was alleged as lightening agent based on its action on amphibian skin.[21,50] Among mammals, melatonin's role in fur pigmentation has been well established[51,52] and subsequently reviewed by Slominski et al.

[3,53] The anti-melanogenic activity was also documented in rodent melanoma cells[54] and murine skin organ culture[45] while its oncostatic activity was elucidated in rodent and human melanomas.[54-56] Numerous reports showed that melatonin induces cell pigmentation in human amelanotic cell lines, that is, Sk-Mel-1, Sk-Mel-23 or Sk-Mel-28.[57,58] Thus, these data may seem to be in conflict to the results of others on melatonin functions in human hair and skin pigmentation.[3,27,59] Locally produced melatonin may play a role in the regulation of melanocytic activities via its impact on the peripheral clock. Therefore, testing of topically applied melatonin during defined circadian windows as an external modulator of intracutaneous clock activity as well as describing the mechanism of action of melatonin in human pigmentation requires still better definition.

To better understand the melatonin's capacity of modulation of melanogenesis, we perform our investigations, contrary to the others, on melanotic cell model as well as in presence of inhibitors of melanin synthesis (N-phenylthiourea (PTU), kojic acid). These results together with latest reports undoubtedly contribute a significant enhancement of knowledge to comprehensively describe the biological meaningful of melatonin and its interaction of complexed mechanism of melanogenesis.

2 | MATERIALS AND METHODS

2.1 | Reagents

Minimum essential medium eagle (MEM) with low glucose (1000 mg/L), 1% penicillin-streptomycin solution (10 000 units of penicillin and 10 mg of streptomycin in 1 mL 0.9% NaCl), acetonitrile, bovine serum albumin (BSA), cacodylate buffer (0.1 mol/L), EDTA, ethanol, formic acid, HEPES (1 mol/L), glucose, glutaraldehyde, HCl, isocitrate, isopropanol, KCl, kojic acid, L-DOPA, lead citrate, leucine enkephalin, melatonin, methanol, methylene chloride, $MgSO_4$, MTT, NADPH, NaCl, NaOH, nonessential amino acids (NEAA) (100×), N-phenylthiourea (PTU), 1% OsO_4, propylene oxide, serotonin, sodium acetate, Triton® X-100, and uranyl acetate were purchased from . Fetal bovine serum, 0.05% trypsin/0.53 mmol/L EDTA solution, 1 × PBS (pH 7.4), L-glutamine (200 mmol/L), AIM-V™ medium were supplied by Thermo Fisher Scientific.

2.2 | Cell culture

Melanoma cell lines included human melanotic MNT-1 cells acquired as a gift from Dr Cedric Delevoye (Institute Curie), and human amelanotic cells (Sk-Mel-28) (American Type Culture Collection). MNT-1 cells were cultured in MEM medium supplemented with 20% (*v/v*) heat-inactivated fetal bovine serum, 10% (*v/v*) AIM-V™ medium, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1% (*v/v*) NEAA, 1% (*v/v*) streptomycin-penicillin solution. Sk-Mel-28 cells were maintained in MEM medium supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 1% (*v/v*) streptomycin-penicillin solution. Both cell lines in the logarithmic growth phase were used in all experiments while 80%-90% monolayers of confluent cells were harvested with a mixture of 0.05% trypsin-EDTA solution.

2.3 | Cell treatment

Prior to beginning the treatments, cells were cultured in normal medium for 24 hours to attach to the bottom of the culture dish. Culture medium was replaced with fresh medium containing melatonin versus control sample, that is., 0.2% ethanol in culture medium. Melatonin was dissolved in absolute ethanol and further diluted with 1 × PBS to yield 10^{-2} mol/L stock solution. Cells were treated with the final concentration of melatonin ranging from 10^{-11} mol/L to 10^{-3} mol/L in time-dependent manner for 24, 48, and 72 hours for cell viability (MTT Assay), for melanin synthesis and DOPA oxidase activity of tyrosinase (melatonin range: 10^{-8}, 10^{-6}, 10^{-3} mol/L for 72 hours) in presence of selected inhibitors, that is, 10^{-3} mol/L PTU or 200 μg/mL kojic acid. The highest resultant concentration of ethanol 0.2% serving as a solvent was not found to be toxic itself to the cells after 72 hours.

2.4 | Cell viability assay

Cells were seeded on 96-well plates at the density of 0.15×10^5 cells/well in the culture medium and incubated with tested compounds until desired time end-point. Cell viability was evaluated by MTT assay based on the protocol described earlier[60] where MTT (5 mg/mL) was dissolved in 1 × PBS, sterilized by filtration through a 0.22 μmol/L Millipore® filter and stored at 4°C. After the treatment, cells were washed twice with 1 × PBS, 100 μL of MTT in culture medium (the final dilution, 1:10) was added to each well, and cells were incubated subsequently for 3 hours at 37°C to allow MTT metabolism. The resultant purple formazan was dissolved in 100 μL isopropanol/0.04N HCl, absorbances were measured at 595 nm using a BioTek EL×808™ microplate reader (BioTek Instruments Inc), and results were normalized to the control cells at 0 hour.

2.5 | Melanin content and electron paramagnetic resonance spectroscopy analysis

Preliminary assessment of melanin content in MNT-1 cells was supported by electron paramagnetic resonance (EPR) spectroscopy.[61] After 72 hours incubation with melatonin in dose-dependent manner (10^{-10}, 10^{-8}, 10^{-6}, 10^{-4}, 10^{-3} mol/L), cells (1×10^6/sample) were pelleted, frozen, and stored at −80°C. EPR analysis was performed in liquid nitrogen using a finger-type quartz Dewar and EMX-AA spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) operating at X-band with 100 kHz magnetic modulation. The amount of melanin in tested samples was determined by comparing double integrals of their EPR signals to the EPR signal of an appropriate melanin standard. For quantification of melanin cells, synthetic DOPA-melanin at the concentration of 0.57 mg/mL was used as described earlier by Sarna et al.[62] Resultant data were presented as content of melanin expressed in pg/cell. Comparatively, cysteine-L-DOPA-melanin at a concentration of 1.34 mg/mL was used to show the characteristics of EPR signal of pheomelanin.

Analysis of suppressive actions by used inhibitors and melatonin on melanogenesis was evaluated as follows: Cells were seeded on 6-well plates (Sarstedt) at the density of 0.3×10^6 cells/well and were allowed to attach overnight. Thereafter, cells were incubated for 72 hours in fresh medium containing various concentrations of melatonin or in mixture with 10^{-3} mol/L PTU or 200 μg/mL kojic acid. For determination of melanin content, the MNT-1 cells were harvested, washed with 1 × PBS, centrifuged at 1000 *g* for 10 minutes (4°C), and solubilized in 500 μL of 1N NaOH for 2 hours at 80°C. The absorbances were measured at

405 nm using a BioTek EL×808™ microplate reader, and results were presented as the percentage of the control sample.

2.6 | DOPA oxidase activity of tyrosinase

MNT-1 cells were seeded on 6-well plates and incubated with melatonin or in a mixture of melanogenesis modulators for 72 hours. Cells were harvested, washed with 1 × PBS, centrifuged at 1000 *g* for 10 minutes (4°C), and lysed with 0.5% Triton® X-100 in 1 × PBS on ice. The lysates were subsequently centrifuged at 16 000 *g* for 15 minutes (4°C), 300 μL resultant supernatant was added to 300 μL of 5 mmol/L L-DOPA in 1 × PBS, and incubated for 1 hour at 37°C. The dopachrome formation was evaluated by measuring absorbance at 475 nm using a BioTek ELx808™ microplate reader, and results were presented as the percentage of the control sample.

2.7 | Liquid Chromatography-Mass Spectrometry (LC-MS) detection of induced production of melatonin

Cells were seeded at the density of 1×10^7, washed with PBS and resuspended with 1 mL HEPES-buffered medium (100 mmol/L HEPES, 120 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L $MgSO_4$, 15 mmol/L sodium acetate, 10 mg/mL BSA and 10 mmol/L glucose; pH 7.4). The reactions were initiated by addition of 100 μmol/L serotonin, 100 μmol/L NADPH, and 1 mmol/L isocitrate. After 24 hours shaking incubation (70 rpm) at 37°C, the extractions were performed twice using 2.5 mL methylene chloride. Resulting samples were dried with liquid nitrogen, redissoved in methanol followed by LC-MS analysis using Xevo G2-XS QTof LC-MS system (Waters). Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm) (Agilent Technology) and Atlantis C18 column (100 × 4.6 mm, 5 μm) (Waters) were used for LC-MS analysis. The flow rates were 0.3 mL/min with isocratic or linear gradient of acetonitrile containing 0.1% formic acid: 15% for 1.5 minute, 15%–30% for 0.1 minutes, 30% for 0.9 minutes, 30%–100% for 0.5 minutes, 100% for 3 minutes for Zorbax Eclipse Plus C18 column, and 0.5 mL/ min with isocratic or linear gradient of acetonitrile containing 0.1% formic acid: 15% for 1.5 minutes, 15%–30% for 3.5 minutes, 30% for 2.5 minutes, 30%–100% for 2.5 minutes, 100% for 5 minutes for Atlantis C18 column. The mass was scanned the range of 100 to 1000 Da in positive mode using the continuum mode with scan time of 1 second. The capillary and cone voltages were 1.7 kV and 40 V, respectively. The desolvation gas flow rate was 800 L/hour with source temperature of 120°C. Leucine enkephalin at the concentration of 200 ng/mL (m/z = 556.2771) was used as the lockspray reference compound at the flow rate of 10 μL/min with lockspray interval of 10 seconds and scan time of 1 second. The mass chromatograms were processed by Waters MassLynx 4.1 software.

2.8 | Transmission Electron Microscopy (TEM)

Briefly, cells were seeded on 6-well plates in the culture medium, and thereafter grown to subconfluence (as judged from light microscopy). Cells were treated with melatonin for 72 hours, harvested, collected by centrifugation (700 rpm. for 5 minutes), washed three times with 1 × PBS, and fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer for 24 hours at 4°C. Cells were washed three times with 0.1 mol/L cacodylate buffer, postfixed in 1% OsO_4 for 2 hours at room temperature (RT), and washed again in distilled water. Cells

were embedded in Poly/Bed®812 (Polysciences Inc) after dehydratation in ethanol and propylene oxide. Ultrathin sections (65 nm thick) were counterstained with uranyl acetate and lead citrate before observation with a Jeol JEM 2100 HT transmission electron microscope.

2.9 | Statistical analysis

Experiments were performed at least three times, with results expressed in each case as the mean + standard deviation (SD). Significant differences between results were determined by the univariate analysis of variance (ANOVA) or the Student's *t*test and appropriate post hoc analysis using GraphPad Prism 7.05 software. Obtained data for viability assay, melanin content, tyrosinase activity were normalized and are presented as percentage of the control sample, that is, 0.2% EtOH. A *P*-value of less than .05 was considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | The effect of melatonin on melanoma cell proliferation

Results of cell viability showed no statistically significant effect of melatonin on cell proliferation in highly pigmented MNT-1 cells, which is in contrast to amelanotic Sk-Mel-28 melanoma cell line Figure 1A,B and inserts). The dose-dependent inhibitory action on Sk-Mel-28 cells was apparent at 48 and 72 hours with the most potent growth inhibition seen at the highest dose of melatonin, that is, 10^{-3} mol/L Figure 1, inserts for 48 hours). These results, involving especially Sk-Mel-28 cells, are in direct agreement with previous reports performed by Souza et al.[58] A similar pattern of regulation upon melatonin in suspension Sk-Mel-1 melanoma cells has been described by Cabrera et al[57] or in other melanoma cell lines, indicating an oncostatic effect of melatonin.[55,63] Other studies demonstrated in vitro anti-proliferative actions of this molecule in amelanotic rodent melanomas, that is, S-91 mouse and Bomirski AbC1 hamster cell lines,[54,64] in human M-6 melanoma cells[65] or Sk-Mel-188 melanoma cultured in Ham's F10 medium.[63] This inhibitory activity is consistent with a large number of investigations showing the ability of melatonin to reduce cancer cell proliferation in lymphoid, prostate, carcinoma, and neuroblastoma tumor cells.[66-73] In contrast to these studies, the melanotic MNT-1 cell line was resistant to significant inhibition of cell proliferation Figure 1A and corresponding insert). We assume that melatonin-mediated arrest of cancer cell proliferation could be connected with the fact that the main target organelles for this indoleamine are mitochondria. Thus, glucose regulation/energy metabolism in cancer cells is critically dependent either on mitochondria or because of fact that most cancer cells use cytosolic glycolysis for ATP synthesis. Moreover, the differences in cancer cell sensitivity to melatonin, which we observed here between MNT-1 and Sk-Mel-28, may also depend on the specific metabolomic fingerprinting of each cancer cell type. Indeed, millimolar concentrations of melatonin decrease S-phase population and trigger apoptosis in colon cancer cells, while the same concentrations only reduce the proportion of cells in G2/M phase in both osteosarcoma and leukemia cells, without any effect on cell death.[74,75] In support of these observations, our data also suggest that those effects depend on the overall metabolic and differentiation state of the cancer cells. It should be added that the balance between oxidative and glycolytic metabolism is maintained by

hypoxia-inducible factor 1 (HIF-1), a transcription factor which mediates adaptive response to changes in tissue oxygenation. It was shown that increase in HIF-1 dependent mRNAs for genes in the glycolytic pathway is accompanied by increases in total protein levels.[76] Thus, it was well-reported that HIF-1-dependent regulation of mitochondrial metabolism may also contribute to the protective effects of ischemic preconditioning.[77] Furthermore, HIF-1 protein has been also found as the one that is regulated during process of melanogenesis what is presented and discussed below.

3.2 | Effect of melatonin on melanogenesis in melanoma cells

Melanogenesis is regulated at the cellular level via the controlling formation of melanosomes, which can be produced in varying sizes, numbers, and densities depending on melanin content. Finally, melanogenesis is involving at the subcellular level intracellular pathways where the gene expression is encoded by the melanogenesis-related enzymes, including tyrosinase, tyrosinase-related protein 1 and 2 (TRP1 and TRP2). It should be noted that although these three enzymes are engaged in the melanogenesis pathway, only tyrosinase is absolutely necessary for melanogenesis, due to its key role in this process. These pathways are initiated by a variety of hormones, including interleukins, interferons, growth factors, and prostaglandins, which determine not only the quantity but also the quality of the synthesized melanin.[78]

Herein, highly pigmented MNT-1 melanoma has been identified as an optimal model to assess the efficacy of melanogenic regulators. This selection is also in agreement with previous reports regarding pigmentation research.[79-83] Recent studies have shown that melatonin inhibits melanin formation in hair follicles of Siberian hamster[51,52] and in rodent melanoma cells.[54,84] Thus, we checked the role of melatonin in regulation of melanogenesis in human malignant melanocytes. As evident from EPR results (Figure 2A-C), MNT-1 cells contained predominantly eumelanin as indicated by the characteristic spectral line obtained for the cells in comparison to synthetic DOPA-melanin used as standard for eumelanin. In addition, EPR analysis revealed a significant decrease of melanin content after treatment with melatonin reaching the level of 2.34 ± 0.01 (10^{-6} mol/L, $P < .05$), 2.22 ± 0.09 (10^{-4} mol/L, $P < .01$) and 2.09 ± 0.07 (10^{-3} mol/L, $P < .001$) pg of melanin per cell compared to the values in untreated melanoma cells (2.72 ± 0.13 pg/cell) Figure 2D. Furthermore, melatonin doses applied in these studies which elicit inhibition correspond to the physiological limits. It has reported mammalian plasma melatonin concentrations which fall within the range of 10-200 pg/mL, that is, approximately 5×10^{-8} mol/L to 1×10^{-6} mol/L. The inhibitory action of melatonin on melanogenesis occurs in the absence of effects upon tyrosinase, generally accepted as the rate-limiting enzyme in melanin biosynthesis.[85] This confirms earlier in vivo findings of Logan and Weatherhead[86] that increased tyrosinase activity does not inevitably result in melanogenesis. This suggests that pigment production can be controlled other than through the regulation of tyrosinase.[54] For instance, Logan and Weatherhead[52] showed that melatonin (10^{-6} mol/L) brings inhibition of melanogenesis independently of applied series of blockers of this process. This may imply that melatonin arrests melanogenesis through a mechanism which operates at some post-tyrosinase step in the melanin biosynthethic pathway. Slominski and Pruski[54] also presented that melatonin at relatively higher doses, those consistent to this study, is acting as a competitive inhibitor

rather than acting through melatonin receptors or via binding sites for a ligand, however, their location remains to be identified.

Indeed, inhibitory action of melatonin enclosed here is in agreement with previous reports using mouse S91 and hamster AbC1 melanoma cells cultured in vitro,[54,63] and it can be coupled with enzymatic or gene expression-related mechanisms. Melatonin at lower (physiologic) concentrations (0.1-10 nmol/L) affects proliferation with no evident actions toward melanogenesis. High doses (>0.10 μmol/L) inhibited the induction of melanogenesis but was either without an effect or even stimulated (100 μmol/L) proliferation. Thus, differential effects of melatonin on cell proliferation and melanin content could indicate that this indoleamine may regulate or modify both processes through different/independent mechanisms. This was confirmed by subsequent analysis where we noticed a similar pattern of regulation regarding significant drop of pigment content at the dose of 10^{-3} mol/L ($P < .05$) Figure 3A. Comparative images of pellets revealed that there was no effect of melatonin on pigmentation in amelanotic Sk-Mel-28 melanoma cell line (Figure 3, inserted images). Furthermore, we assessed the influence of melatonin's responsiveness to alterations in melanogenesis mediated by selected inhibitors, that is, PTU and kojic acid. They decreased melanin content after 72 hours by 52% and 39% ($P < .001$), respectively Figure 3B,C what was visualized afterward using the light microscope Figure 3D. These data are consistent with PTU-treated Sk-Mel-188 melanomas presented by Brożyna et al[87] or Slominski et al.[88] Interestingly, co-incubation of melatonin indicates an antagonizing effect of neurohormone, however, the difference was only slightly pronounced. Similar action of melatonin was observed at the level of tyrosinase activity Figure 4A,B as well. Presence of inhibitors decreased it by 45% and 26% ($P < .001$) for PTU and kojic acid, respectively, while melatonin antagonized these alterations, particularly at the dose of 10^{-3} mol/L. Above results implicate a logical mechanisms of melanogenesis but clearly suggest to be more complex than originally thought, involving diverse molecular pathways. Slominski et al[47] using combined in vitro and in vivo data demonstrated that stimulation of melanogenesis increases the overall expression of HIF-1α and its subsequent nuclear localization. It is also possible that HIF-1α is induced by the production of intermediates of melanogenesis, including ROS and the consumption of intracellular oxygen by the melanogenic process.[2,3] Indeed, the authors presented that expression of HIF-1α, but not HIF-2α, protein is prominently up-regulated proportionally to induction of melanogenesis in Bomirski hamster AbC1 and human Sk-Mel-188 melanoma cells.[47] As a result, initiation of melanogenesis affects the expression of multiple genes involved in the regulation of melanocyte/melanoma behavior, including the metabolic switch to glycolysis cooridinated by HIF-1. It accompanies the changes in mitochondrial stress-related genes, immunity, angiogenesis, and cell proliferation. This can be linked with our results where we observed significant decrease of melanin synthesis at 1 mmol/L melatonin while Park et al[89] detected inhibition of HIF-1α protein at the same dose of this indoleamine. This shows the correlation between melatonin, HIF-1, and melanogenesis.

Despite all, we suggest that melatonin could be considered as a regulator of pigmentary disorders. Although it is generally agreed that melatonin exerts a hypopigmenting action on most biological systems, its effects in mammalian pigmentation are particularly uncertain and undoubtedly multifaceted. For instance, orally applied melatonin has no noticeable

effects on mammalian pigmentation[90] especially in man.[59] On the other hand, melatonin plays a key role in the control of pigmentation in certain rodent species that undergo seasonal fur color changes.[91] Thus, the synthesis of melatonin is inhibited by long days and is maximal under short days.[92] However, whether the effect of melatonin is directly exerted on melanocytes or indirectly via an interaction with the pituitary synthesis and release of α-MSH, remains to be clarified.

Earlier in vitro analysis showed that MNT-1 cell line contains abundant melanosomes at all stages (ie, stages I–IV)[80] whereas Sk-Mel-28 cells have only stage I and II melanosomes.[93,94] In fact, both, stage I and II melanosomes are early melanosomes or premelanosomes because they have not initiated melanin synthesis. In contrast, stage III melanosomes are characterized by the active synthesis of melanin, which results in the deposition of black electron-dense pigment on a fibrillar matrix. Finally, stage IV melanosomes are fully mature and little internal structure is visible because they are completely packed with melanin. According to Chen et al,[80] MNT-1 cells have relatively even distribution of melanosomes according to stage (II-III to IV),this is in line with our observation conducted by TEM assessment where we showed distinguish stages of developed melanosomes Figure 5. It was also apparent that 10^{-3} mol/L melatonin induced a marked development of premelanosomes at stage I-II Figure 5D with simultaneous maintenance of matured melanosomes at stage III-IV for melatonin at lower doses of 10^{-8} mol/L or 10^{-6} mol/L Figure 5B,C. Endogenous melatonin in MNT-1 cells is also present as we confirmed using LC-MS analysis (Figure 6A-F). This could alleviate our concerns that treatment with melatonin may not be more apparent (masking effect) or is obvious only at high concentration (10^{-3} mol/L). Furthermore, this assessment is that currently investigated melanomas have a significant capacity to synthesize melatonin de novo upon exogenous application of 100 μmol/L serotonin. Thus, micromolar concentration of serotonin is present in serum, therefore, not spectacular effect of serotonin on melatonin production could be related to oversaturated endogenous system. We are aware that our approach regarding evaluation of ability of endogenous synthesis of melatonin in MNT-1 cells is undoubtedly a milestone. Thus, the analysis has defined this cell line as a target model related to the widely understood studies about melatonin and its relevance to melanogenesis.

In conclusion, our study provides evidence that melatonin exhibits anti-proliferative actions in MNT-1 or Sk-Mel-28 cells which are consistent with earlier reports with other melanoma cells.[54,55,57,58,63-65] Collectively, the findings enrich current knowledge where melatonin is an effective antitumor compound useful in the treatment of melanoma alone or in combination with known chemotherapeutic agents to improve the efficacy of conventional cytotoxic agents. Additionally, melatonin possesses multifaceted effects with regard to regulation of melanogenesis. It lightens highly pigmented MNT-1 cells what was not performed so far. It neutralizes pigmentation disturbances mediated by exogenously applied melanogenic inhibitors. The molecular mode of action of melatonin may be related to the fact that it tends to maintain cellular homeostasis as observed earlier when it attenuates oxidative stress,[28,30,31,34,40,95], apoptosis[29,30] and inflammation[33,35] in normal cells while stimulating it in cancer cells. Herein, melatonin's action on pigmentation opens new regulatory targets for this molecule. However, further examinations of these associations are strongly desired, including studies on primary melanocytes. The ability of melatonin to

control this process may serve as a rationale strategy for treatment of pigmentary disorders with this multifunctional molecule.

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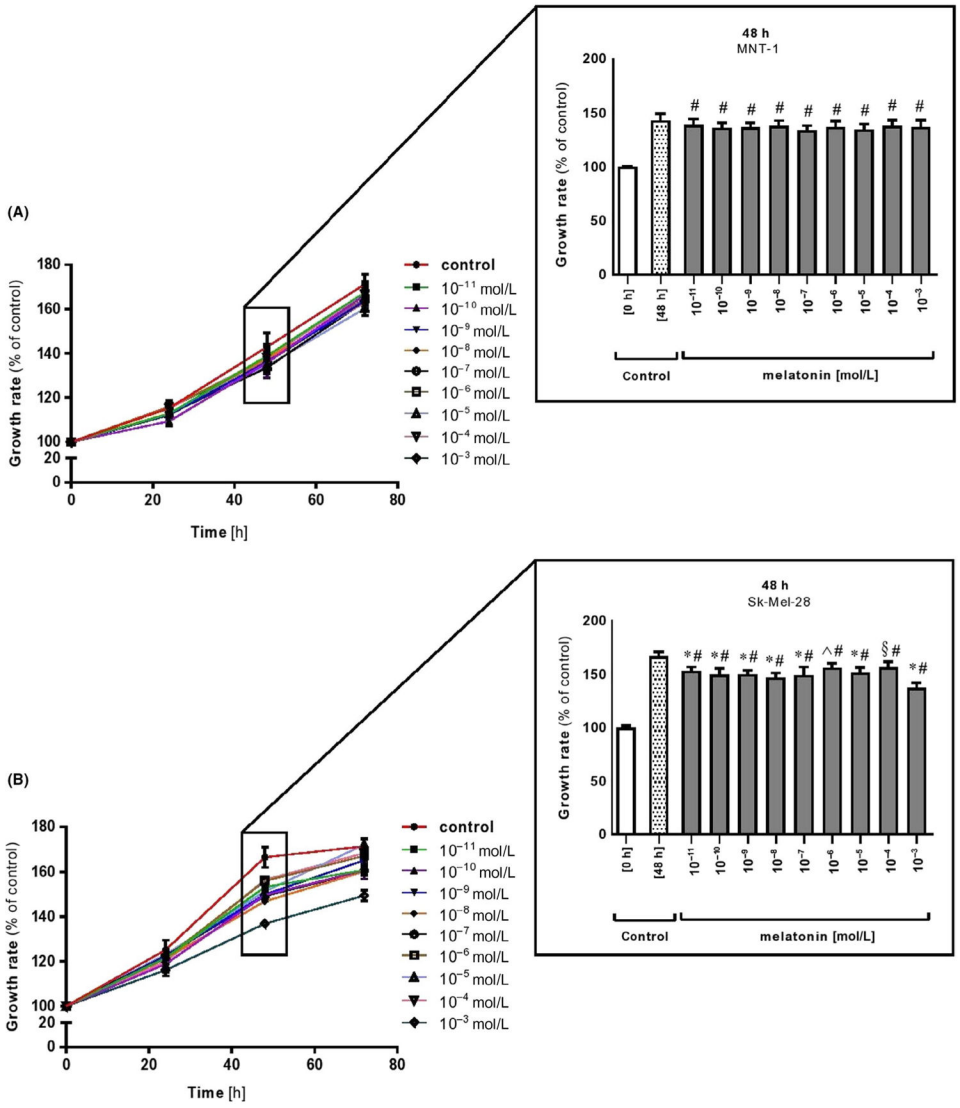

FIGURE 1.

Melatonin displays oncostatic-like effects in melanoma cells. Highly pigmented MNT-1 (A) and amelanotic Sk-Mel-28 (B) melanoma cells were treated with melatonin in dose- (10^{-11}-10^{-3} mol/L) and time-dependent manner (0, 24, 48, 72 h) and were investigated using the MTT viability assay as described in Materials and Methods. Inserts present inhibitory growth rate at selected time end-point, that is, 48 h for MNT-1 and Sk-Mel-28 melanomas. Data were presented as the mean + SD (n = 6), the values are expressed as percentage of the control sample. Statistically significant differences versus control at 48 h were indicated as $^{\S}P < .05$, $^{\wedge}P < .01$, $^{*}P < .001$ while comparison of melatonin-treated cells versus control sample at 0 h was indicated as $^{\#}P < .001$

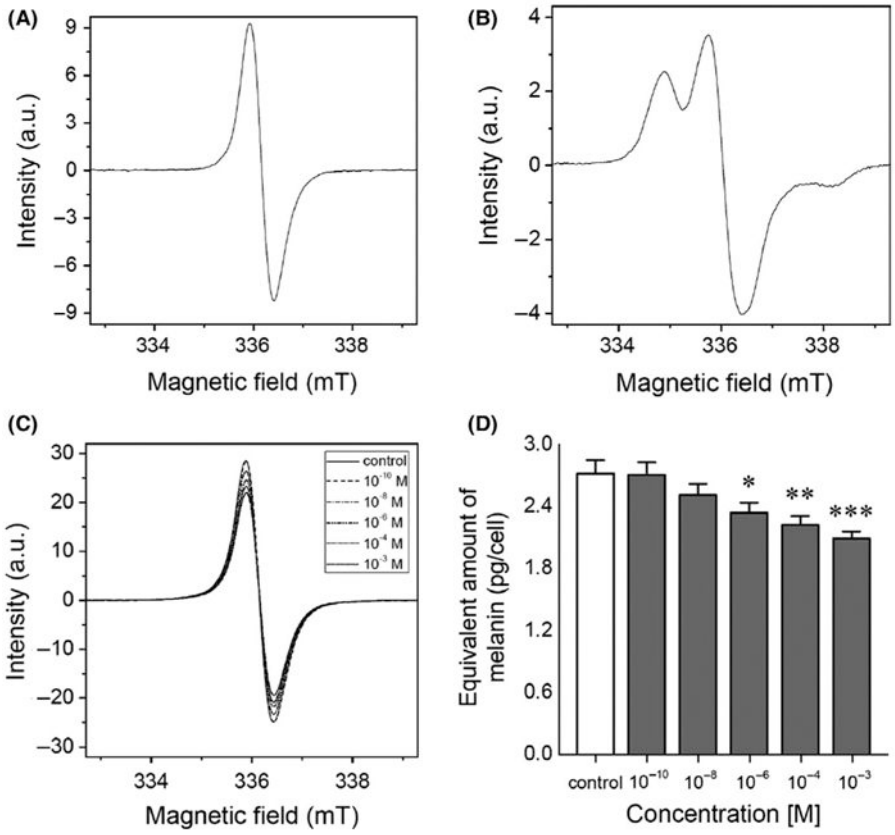

FIGURE 2.

Melatonin decreases melanin content in MNT-1 melanoma cells. Based on assessment carried out by electron paramagnetic resonance (EPR) spectroscopy, here the spectra of (A) synthetic DOPA-melanin used as standard for melanin determination; (B) synthetic cysteine-L-DOPA melanin indicating the characteristic of EPR signal of pheomelanin, and cells after 72 h incubation with melatonin in dose-dependent manner are depicted (**C**). Bar graph displays mean values + SD (n = 3) of pg of melanin per cell in melatonin-treated cells (**D**). Statistically significant differences versus control were indicated as $*P < .05$, $**P < .01$, $***P < .001$

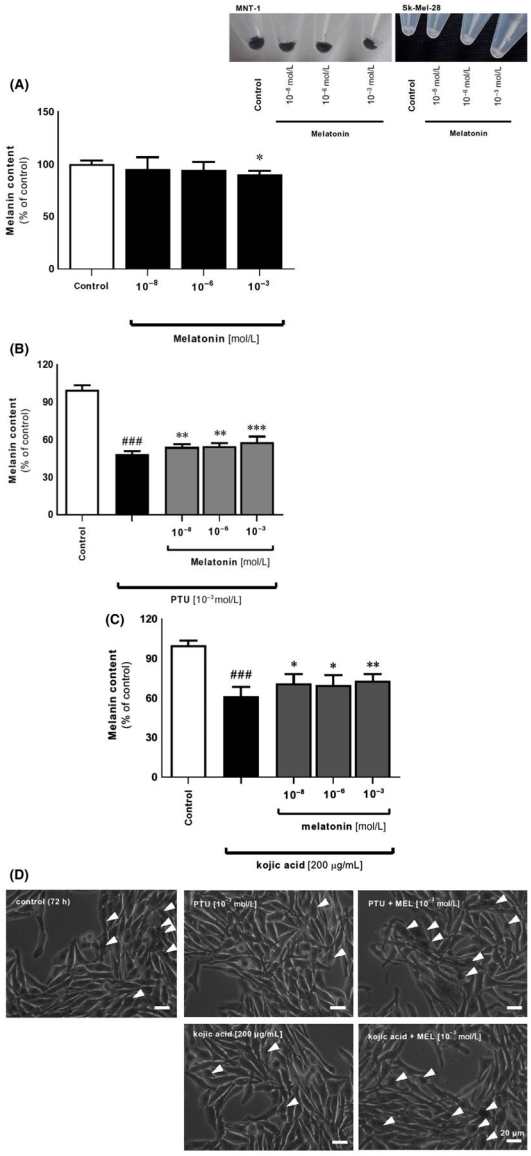

FIGURE 3.

Melatonin counteracts the inhibitor-mediated alterations in cell pigmentation in MNT-1 cells. Images enclosed as insert present distinct differences in pigmentation ratio between MNT-1 and Sk-Mel-28, representatives for highly pigmented and amelanotic cells, respectively. (A) Evaluation of melanin content has been carried out using melanotic MNT-1 cells cultured in MEM supplemented medium for 72 h with melatonin (10^{-8}, 10^{-6}, 10^{-3} mol/L) and melanogenic inhibitors, that is, 10^{-3} mol/L PTU (B) or 200 μmol/L kojic acid (C) as described in Materials and Methods. Data were presented as the mean + SD (n = 5), the values are expressed as percentage of the control sample. Statistically significant differences were indicated as $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ while comparison of PTU- or kojic acid-treated cells versus control sample was indicated as $^{\#\#\#}P < .001$. (D) Visualization of MNT-1 cells in culture and effect of melatonin versus PTU or kojic acid. Arrowheads point out pigmented cells

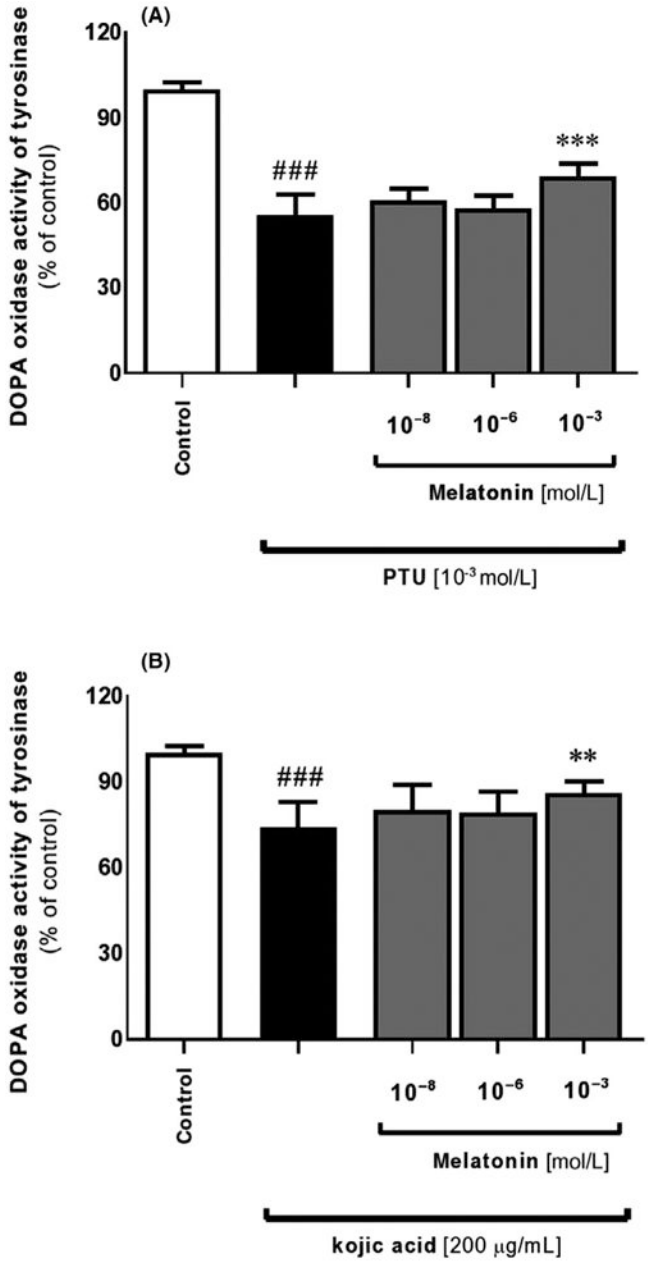

FIGURE 4.

Actions of melatonin on altered tyrosinase activity due to selected inhibitors in MNT-1 melanoma cell line. Applied 10^{-3} mol/L PTU (A) or 200 μmol/L kojic acid (B) significantly affected tyrosinase activity compared to co-incubated cells with melatonin for 72 hours. Briefly, cells were lysed with 0.5% Triton® X-100, centrifuged at 16 000 *g* (15 min, 4°C), 5 mmol/L L-DOPA was added to resultant supernatant, and the dopachrome formation was evaluated by measuring absorbance at λ = 475 nm. Data were presented as the mean + SD (n = 5), the values are expressed as percentage of the control sample. Statistically significant differences were indicated as $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ while comparison of PTU- or kojic acid-treated cells versus control sample was indicated as $^{\#\#\#}P < .001$

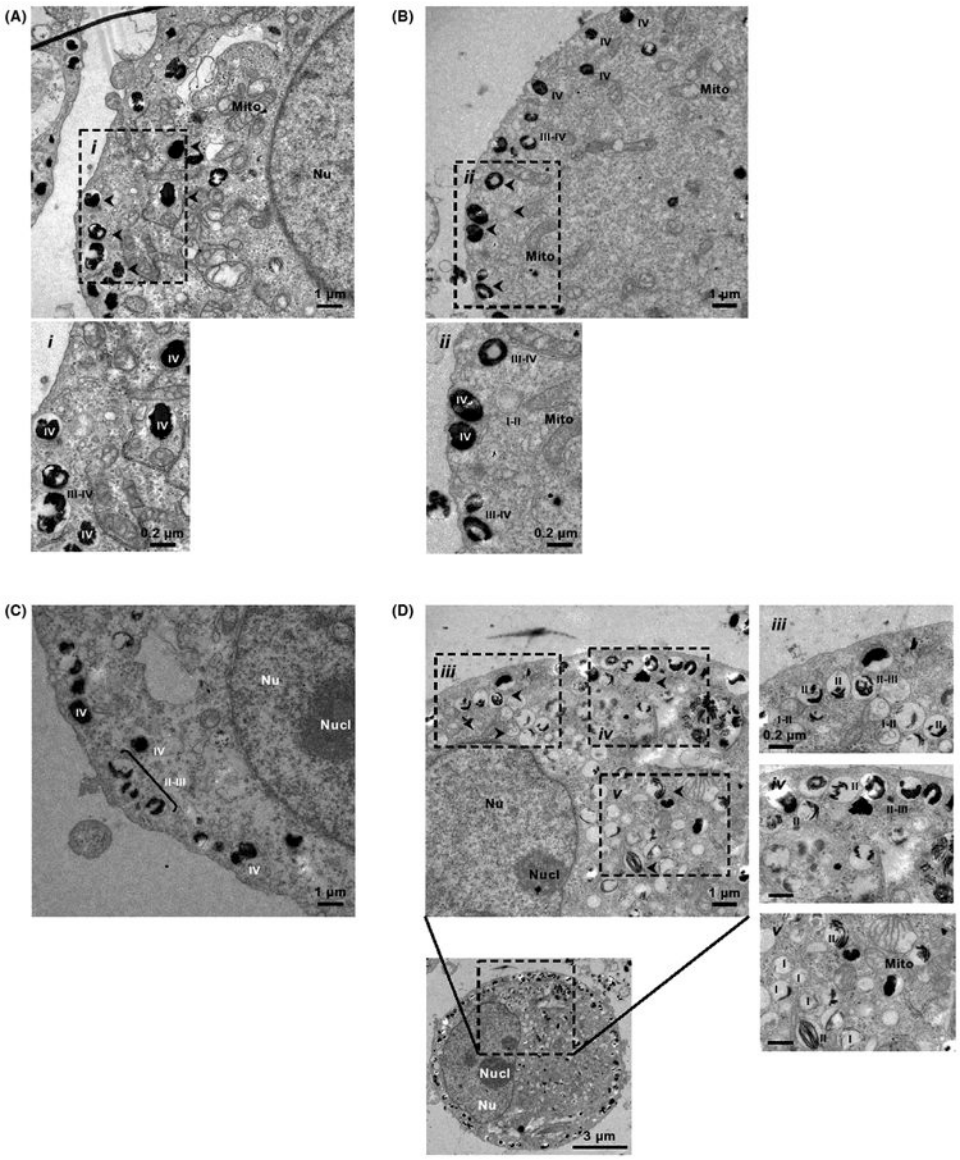

FIGURE 5.

Transmission electron microscopy images were obtained as described in Materials and Methods. This study reveals differences in number of the particular stages of melanosome occurred after 72 hours with melatonin in dose-dependent manner, that is, 10^{-8} mol/L (B), 10^{-6} mol/L (C), 10^{-3} mol/L (D) compared to the control sample (A). Arrowheads indicate some stages of I-IV melanosomes. Nu, nucleus; Nucl, nucleolus; Mito, mitochondria

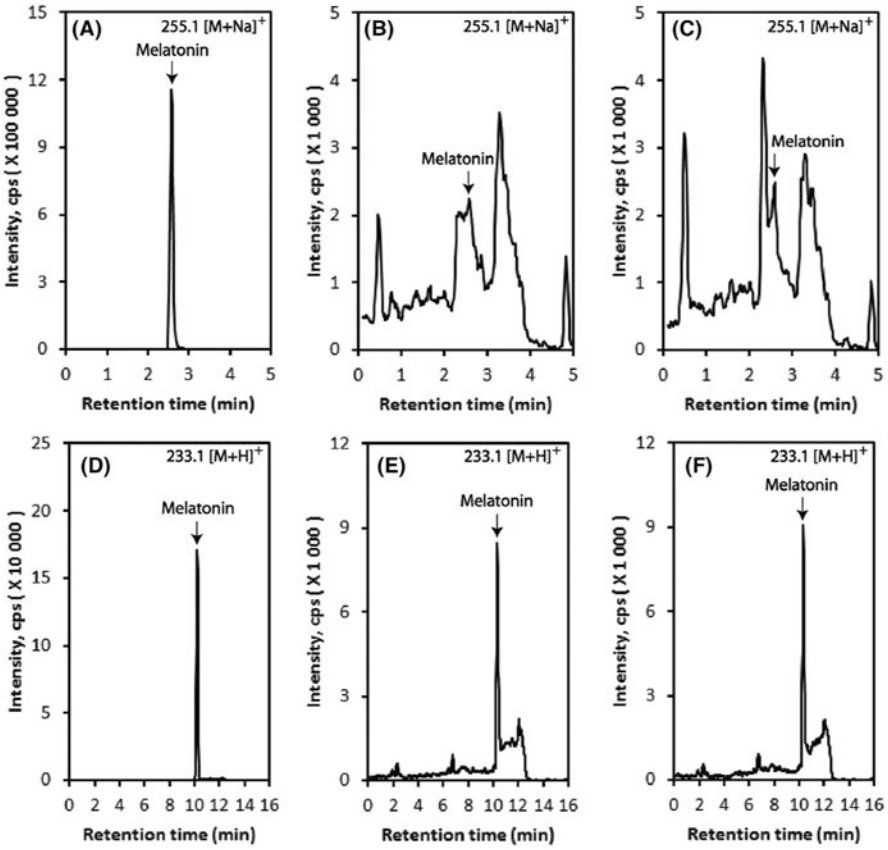

FIGURE 6.

Melatonin production is slightly elevated by endogenous addition of serotonin. Highly pigmented MNT-1 cells were incubated with 100 μmol/L serotonin for 24 hours, and melatonin was extracted with methylene chloride followed by LC-MS using Xevo G2-XS QT of LC-MS system as described in Materials and Methods. Extracted Ion Chromatograms (EIC) with m/z = 255.1 $[M + Na]^+$ using Zorbax Eclipse Plus C18 column are shown: (A) melatonin standard; (B) control (without serotonin); (C) 100 μmol/L serotonin. EICs with m/z = 233.1 $[M + H]^+$ using Atlantis C18 column are shown: (D) melatonin standard; (E) control (without serotonin); (F) 100 μmol/L serotonin