Research Article

Melatonin inhibits growth of B16 melanoma in C57BL/6 mice

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ABSTRACT

Melatonin (N-acetyl-5-methoxytryptamine) has oncostatic properties in a wide variety of tumors. In melanoma, melatonin displayed growth suppressive effects in cultured cell lines and tumors. Thus far, however, there is no evidence of orally administrated melatonin reducing melanoma tumor growth. Therefore, the current study investigated the preventive effect of melatonin on C57BL/6 mice injected with B16-F10 murine metastatic melanoma cells. The animals were divided into two groups; control (vehicle) and melatonin pre-treated with oral melatonin in the drinking water (10 mg/kg/day) for 15 days. Grossly, the control animals had a significant exponential increase in tumor size until day 33, and all control animals were dead by day 38; conversely, melatonin pre-treated mice demonstrated delayed tumour appearance as well as decreased tumour volume and increased survival rates. PCNA immunostaining corroborated these data and demonstrated a significant reduction in the number of proliferating cells in the melatonin-treated mice ($P < 0.005$). Interestingly, histopathological analysis revealed the presence of undifferentiated and pleomorphic cells associated with higher mitotic rate in the control group, while epithelioid-shaped cells, sometimes containing melanin were clearly identified in melatonin-treated animals. Mitochondrial parameters measurement showed greater PTP opening and increased mitochondrial nitrite level associated in melatonin-pretreated animals. Finally, the decreased P-ERK1,2 cytoplasmic expression in melatonin mice compared with the controls supports the conclusion that the MAPK signalling pathway is repressed by melatonin in B16-F10 melanoma. Collectively, these results suggest for the first time that orally-administered melatonin reduces malignant melanoma progression *in vivo* and increases the percent of survival by lowering tumor cells proliferation due to mitochondrial dependent cytotoxicity and decreased P-ERK1,2 expression. This study demonstrates the chemopreventive potential of melatonin against malignant melanoma in C57BL/6 mice.

Key words: Melanoma, melatonin, C57BL/6 Mice, MAPK signalling pathway.

1. INTRODUCTION

Malignant melanoma is a highly aggressive cancer, and causes high mortality because of rapid growth, metastasis, and resistance to chemotherapy (1). Worldwide, the incidence of malignant melanoma is increasing, and is currently rising more rapidly than any other cancer (2). In 1930, the lifetime risk of an American developing invasive malignant melanoma was 1 in 1,500. In 2010, that risk was 1 in 59, moreover, deaths from malignant melanoma are also increasing (3). Many therapeutic approaches to suppress melanoma growth and metastasis have been attempted (4-5). *In-vitro* melatonin has been shown to suppress the growth of cancer cells, including malignant melanoma (6).

Melatonin is an indoleamine produced at night mainly by the pineal gland and also locally in many other tissues (7, 8). Pineal melatonin mediates the photoperiodic entrainment of endogenous circadian rhythms (9) and is also involved in numerous other functions, such as antioxidant (10-11), immunomodulatory , antiaging (14) and tumor inhibition (15-18).

Oncostatic properties of melatonin is involving interfering with recent of the cancer hallmarks and influencing different pathways with direct effects on the cell cycle, antioxidant and immunostimulatory actions on the neoplastic process (17, 19). Many oncogenic pathways that cause melanoma tumorigenesis have been identified among which the mitogen activated protein kinase (MAPK) signaling pathway was found to play a critical role in melanoma initiation and metastasis (20-21). More recently, the p38α-MAPK specially, was shown to be a major kinase effecting melanoma development and progression by inducing key angiogenic and EMT factors expressions (22).

Several studies reported on the anti-tumor actions of melatonin against malignant melanoma both *in vitro* or *in vivo* (6, 16, 23-28). Thus, the present study was undertaken to assess the ability of orally-administered melatonin, an otherwise non-toxic chemoprotective agent against malignant B16-F10 melanoma cells subcutaneously injected into C57BL/6 mice. Endpoints included proliferative activity, differentiation status, mitochondrial dependent cytotoxicity and MAPK signaling pathway activation in the *in vivo* growing melanoma tumor cells.

2. MATERIALS AND METHODS

2.1. Animals.

Experiments were performed in female C57BL/6 mice (Harlan, Udine, Italy) (IMSR Cat# JAX 000664, RRID:IMSR JAX:000664) weighing 18-20 g (29). Animals were housed under a 12/12 h day/night cycle in temperature-controlled rooms (22 ± 2 ºC) and were fed a standard laboratory diet (Harlan Teklad Research Diet, Madison, WI, USA) and tap water *ad libitum*. Animals were allowed to acclimate for one week before experimental handling. Animal care and protocol were carried out in accordance with institutional (Research Ethics Committee of the University of Granada, Spain) and international standards (European Communities Council Directive 86/609; protocol number: 1 03-2016-CEEA).

2.2. Experimental protocol.

Animals were subdivided into two groups of 12 animals each. Vehicle-treated animals served as control group, and the other treated group received a pre-treatment with melatonin (10 mg/kg) for 14 days. After that, both animal groups were injected with tumor cells and

treatment with vehicle and melatonin, respectively, continued until the end of the study (day 38). Vehicle (0.066% ethanol) and melatonin were administered in the drinking water *ad libitum*. Melatonin-treated were followed the study until all animal died (97 day).

2.3. Tumor growth and survival.

Tumors were induced by subcutaneous injection of 2 x 10^5 B16-F10 cells (Cat# CSC-C9336L, RRID:CVCL_XH27) into the right flanks of C57BL⁄6 mice. Tumors were allowed to grow (around 75 mm^3 which is the optimum minimal size for intratumoral injection) before treatment and then they were measured at periodic intervals using a digital caliper by measuring the longest diameter (a) and the next longest diameter (b) perpendicular to (a) according to a previously-established methodology. The tumor volume was calculated by the formula $V = ab^2 \times \pi/6$ (mm^3) (29). The tumor growth curve was constructed based on tumor size. In our experimental conditions all flasks were ≤75% confluent when harvesting the cells. Under these conditions, 10-15 days was adequate time to establish a measurable tumor size. Cells were routinely tested for negative mycoplasma contamination by our Biobank service of the Bioresearch Center. Survival was evaluated in mice bearing xenografts until spontaneous death. The survival curves were performed by Kaplan–Meier method.

2.4. Mitochondrial isolation.

Tumors were removed, excised, washed with cold saline, and homogenized in isolation medium (10 mMTris, 250 mM sucrose, 0.5 mM Na_2EDTA, and 1 g/L free fatty acid BSA, pH 7.4, 4ºC) with a Teflon pestle. Mitochondria were isolated by serial centrifugation. The homogenate was centrifuged at 1,000 g for 10 min at 4ºC and the supernatant was centrifuged again at 15,000 g for 20 min at 4ºC. The resultant pellet was resuspended in 1 mL of isolation medium without BSA, and an aliquot was frozen for protein measurement. The remaining mitochondrial suspension was centrifuged at 15,000 g for 20 min at 4ºC and resuspended in 1 mL of respiration buffer (20 mM HEPES, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 200 mM sucrose, and 1 g/L fatty acid free BSA). Mitochondrial suspensions were kept on ice for 10–15 min before starting the experiments to permit the rearrangement of the membrane. To prepare submitochondrial particles, mitochondrial pellets were frozen and thawed twice, sonicated, and suspended in the corresponding medium. The protein concentration was 0.2-0.4 mg protein/mL for each assay.

2.5. Mitochondrial respirometry.

Oxygen consumption was measured with a high resolution oxygraph (Oroboros oxygraph-2k equipment, Oroboros Instruments, Innsbruck, Austria), consisting in a two chambers respirometer with a peltier thermostat and electromagnetic stirrers (30). The oxygraph was constructed to minimize oxygen back-diffusion by using impermeable materials for oxygen (30). Analysis of respiration was performed in 2 mL of respiration medium at 30ºC. The medium was previously equilibrated in each chamber with air at 30ºC and stirred at 750 rpm until a stable signal at air saturation was obtained. A final concentration of 0.2–0.3 mg/mL fresh proteins of mitochondria in the respiratory buffer was used for the experiments. Mitochondria were suspended in incubation medium supplemented with glutamate (5 mM)/malate (2.5 mM) or with succinate (5 mM), as energizing substrates in the presence of rotenone Jo2 was recorded at 30ºC in a constantly stirred oxygraph vessel after successive additions of 1 mM ADP (state 3), 0.75 mM oligomycin (state 4), and 75 mM DNP. State 3 of respiration (consumption of oxygen in the presence of substrate and ADP) was initiated with

ADP (75 nmol/mg protein). States 3 and 4 (consumption of oxygen after ADP phosphorylation) of respiration. The protocol completed within 15-20 min, was recorded at 0.2 s intervals using a computer-driven data acquisition system (Datlab, Innsbruck, Austria).

2.6. Enzyme assays.

Complex Ispecific activity was measured by following the loss in absorbance due to the oxidation of NADH at 340 nm (31). Submitochondrial fractions (0.3 mg/mL) were incubated for 5 min in a medium containing 250 mM sucrose, 50 mM potassium-phosphate, 1 mM KCN, 0.5 mM decylubiquinone, pH 7.4. The reaction was initiated by the addition of NADH.

Complex III specific activity was measured by monitoring the reduction of cytochrome c at 550 nm, in a medium containingsubmitochondrial particles (0.03 mg/mL), 0.1 M potassium-phosphate, 15 mM decylubiquinone, 0.1 M NaN_3 and BSA (10%), pH 7.5. The reaction was started by adding oxidized cytochrome c (1%) (32-33).

Complex IV activity (nmol oxidized cytochrome c/min/mg protein) was determined in a medium containing 75 mM potassium-phosphate pH 7.4 at 25 ºC. The reaction was started by the addition of cytochrome c previously reduced with sodium borohydride (31, 34). The activity was measured as the disappearance of reduced cytochrome c at 550 nm.

2.7. Determination of mitochondrial nitrites.

This method involves the use of the Griess diazotization reaction to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under physiological condition (35). This determination was carried out after mitochondrial isolation, using a Griess Reagent Kit (Molecular Probes, G-7921), with optimum measurement wavelength of 548 nm as described in manufacturer instructions.

2.8. Mitochondrial activity of SOD.

The superoxide dismutase (SOD, both Mn and Cu/Zn) activity was measured using a SOD assay Kit-WST (Sigma-Aldrich, 19160, Switzerland), after mitochondrial isolation, following the kit instruction. Results are expressed as inhibition rate percentage (SOD activity). The minimum detectable amount for SOD activity was 0.001 U/ml.

2.9. Calcium retention capacity.

The sensitivity of the mitochondrial PTP to calcium was evaluated fluorimetrically by monitoring the calcium retention capacity of mitochondria supplemented with 0.25 μM Oregon green. Pulses of 0.50 μM Ca^{2+} were added until pore opening, and the specificity was assessed by adding 1 μM Cyclosporine A, the standard inhibitor of PTP (36). The mitochondrial PTP opening was calculated from the area under the curve of fluorescence signals over time.

2.10. Histopathological examination and mitotic rate assessment.

Tumors were excised and directly fixed in formalin 10%. Samples were dehydrated in alcohol and cleared in xylene and then embedded in paraffin. The samples were cut at 4 μm thickness. The slides were stained by H&E. Mitotic rate was assessed by counting mitotic figures in ten X400 HPF and expressed as the mean ± S.E.M. Vascularity was determined as the number blood vessels per 1 mm^2 according to Burton *et al.* (37).

2.11. Immunohistochemical staining.

Samples were taken from all animals, and were blindly examined for semiquantitative estimation for degeneration, necrosis, melanin and morphology of the cells. Serial sections of tumor tissue were dewaxed, hydrated, and immersed in an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) for 15 min and autoclaved at 121°C and100 °C for 15 and 10 min. They were then treated with hydrogen peroxide 0.3% and protein block, followed by incubation with mouse monoclonal PCNA antibody (clone PC10; 1: 200, Catalog number [M0879], Dako, Glostrup, Denmark) at 4 °C overnight. The slides were rinsed three times with PBS, incubated with anti-mouse IgG secondary antibodies for 30 min at room temperature, visualized with di-aminobenzidine commercial kits, and finally counterstained with Mayer's haematoxylin. To get average positive level of each animal, only a distinct brown-colored nucleus was accepted as a positive reaction and about 10 microscopic fields of X400. PCNA-LI is the percentage of immunopositive cells per total 1000 neoplastic cells. Quantitative scoring of mitosis was estimated as the count of mitotic figures in 10 HPF and expressed as mean ± S.E.M. For immunohistochemistry, PCNA was determined by counting the total positive cells in 1000 neoplastic cells and expressed as percent of positive cells. While ERK1/2 was analyzed through photographing about 30 images which were analyzed by ImageJ software (version 1.32j, NIH, USA); these data were expressed as percent of positive area.

For immunolabeling of phosporylated ERK 1 and 2, serial sections were dewaxed, hydrated, washed in TBST, and then immersed in 10 mM sodium citrate buffer, pH 6.0, maintained at a sub-boiling temperature for 10 min and cooled on bench top for 30 min. After inactivation of endogenous peroxidase and blocking of non-specific binding of the antibody, slides were incubated with rabbit monoclonal Phospho-p44/42 MAPK (Erk1/2) primary antibody (1:400 dilution; Catalog number (4370) Cell Signaling, Technology, Beverly, MA). The sections were incubated for 30 min in goat anti-mouse HRP-antisera, and then remaining procedures were performed in the same manner as PCNA immunolabeling.

2.11. Statistical analysis.

The data were analyzed using the GraphPad Prism version 7 (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as means ± S.E.M. Comparison between the two experimental groups was analyzed by using Mann-Whitney U test to compare the mean values between two groups. In case of survival curve, the data were analyzed by the log-rank test. *P* value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Survival percent and tumor volume.

The effect of melatonin to inhibit growth of B16-F10 melanoma tumors in female C57BL/6 mice was evaluated. The tumors were palpable at ten days post-inoculation. On day 14, the tumors of control mice were readily apparent. Then, significant exponential growth was noticed until day 33. Therefore, the percent survival decreased in a time-dependent manner and nullified (all animals died) at day 38 in non-melatonin treated control group. However, in the group pre-treated with melatonin, the percent survival remained at 100% until day 33 and reached 75 % on day 38 (Figure 1A). In melatonin pre-treated mice, the tumor growth had a significant latency period of almost two weeks (on day 25) more than in the control group. Also, the tumor size was significantly less on days 22, 25 and 33 with respect to control group; however, by day 38 post-inoculation, they were non-significantly different (Figure 1B).

Nevertheless, it should be taken into account that the growth rate slopes, once a tumor was measureable in melatonin treated group, are similar for both groups. These results document an inhibitory effect of orally administered melatonin on B16-F10 melanoma appearance and overall survival *in vivo.* Additionally, the pattern of decreased percent survival was highly significant ($r^2 > 0.95$) and inversely correlated with increased tumor volume (Figure 1C).

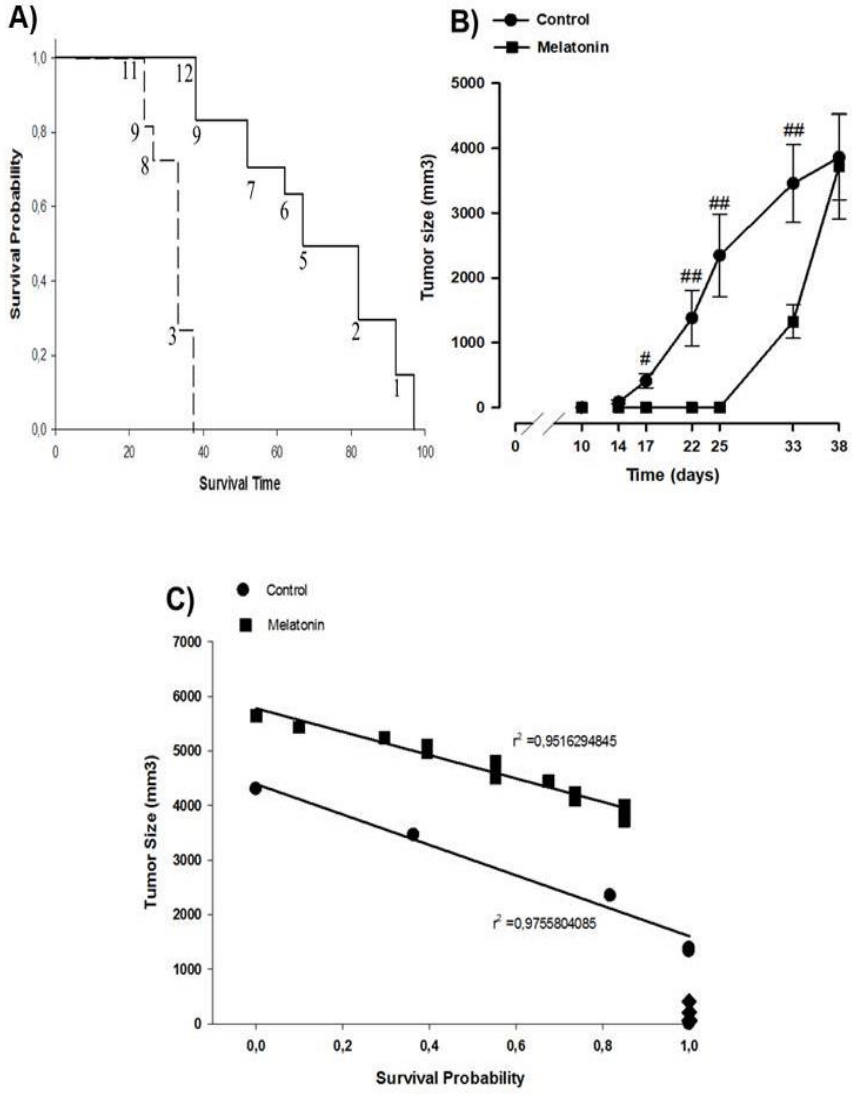

Fig. 1. Effects of melatonin survival percent and tumor volume.

(A) *Effect of melatonin on the survival of animals over time. Kaplan-Meier curve for overall survival of NSG mice injected with 2 x 10^5 from B16-F10 cells into the right flanks of C57BL/6 mice.* ***(B)*** *Tumor volume (mm^3) of subcutaneously xenografted tumors formed by 2 x 10^5 from B16-F10 cells in C57BL/6 mice. Data are shown as mean ± S.E.M. (**P < 0.01; *P < 0.05).* ***(C)*** *Correlation between tumor size and survival probability.*

3.2. PCNA expression is reduced in melatonin-treated tumors.

To corroborate the difference in tumor growth between melatonin-treated and control groups, we compared their expression levels of the proliferating cell nuclear antigen (PCNA) protein. Melanoma metastasis is a complex process (38). PCNA plays an important role in the cell proliferation as an auxiliary protein for DNA polymerase delta (39-40). PCNA stain is one of many markers of proliferative potential of melanoma (41). Our results showed an obvious

reduction in PCNA protein expression in tumors sections from melatonin-pretreated animals compared to the control (Figure 2C and D). Our findings justified that the diminishment of PCNA in melatonin-pretreated group could be associated to a lower melanoma metastasis.

On the other hands it could very interesting in near future to study if melanoma tumors of melatonin-treated animals could become refractory to melatonin like many tumors become refractory to chemotherapies.

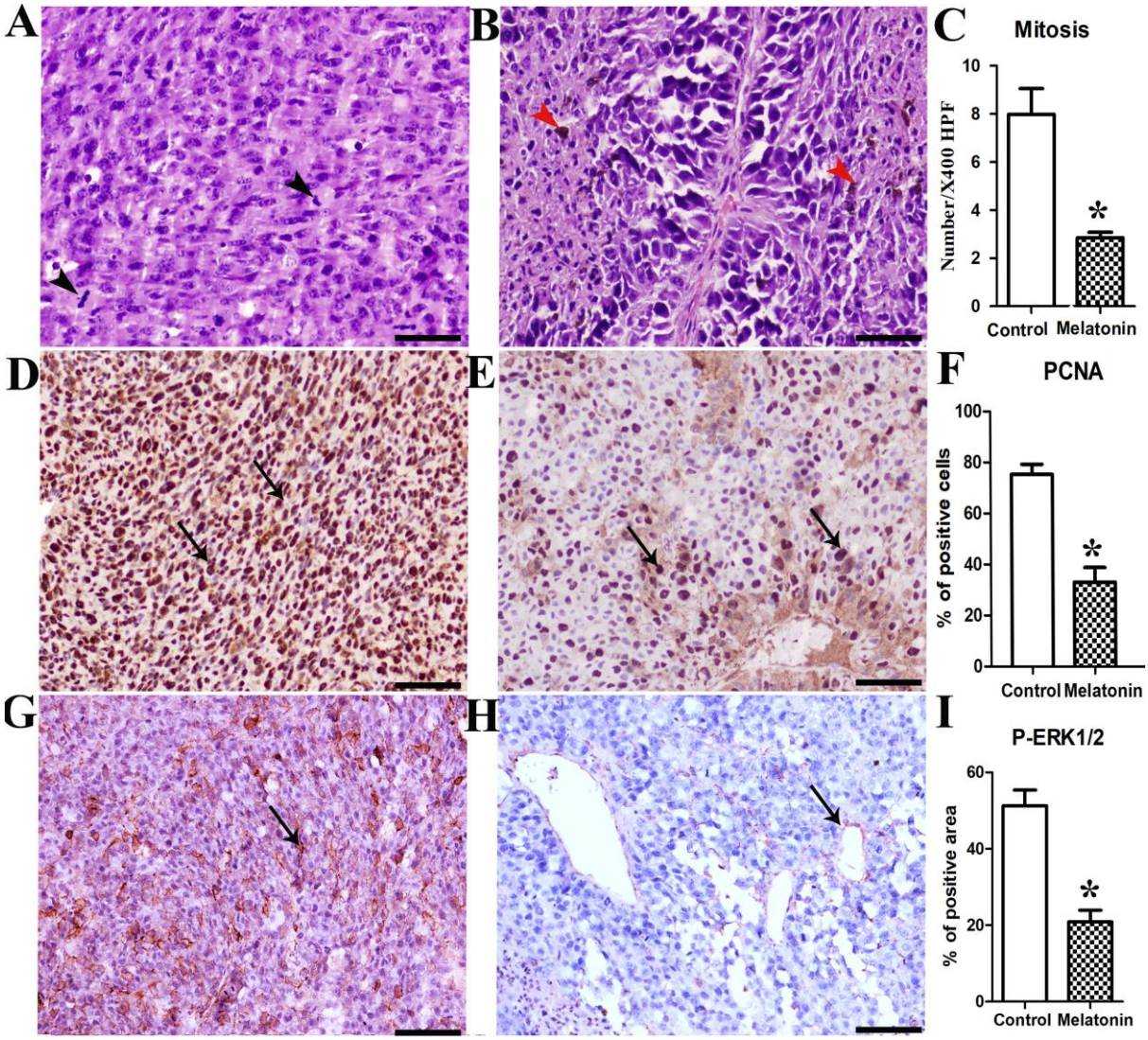

Fig. 2. Effects of melatonin on PCNA expression.

(A) *Pleomorphic neoplastic cells with high mitotic index (black arrowheads),* ***(B)*** *Perivascular epithelioid neoplastic cells associated with presence of pigmented cells (red arrowheads) and marked necrosis,* ***(C)*** *significant decrease of mitosis in melatonin mice (P < 0.01).* ***(D, E*** *and* ***F)*** *representative PCNA immunostaining of control, melatonin-pretreated groups and PCNA-LI, respectively.* ***(G, H*** *and* ***I)*** *Representative phosphorylated ERK 1 and 2 immunostaining of control, melatonin-pretreated groups and P-ERK1/2 LI respectively,* ***(G)*** *Increase in cytoplasmic expression,* ***(H)*** *Showed weak expression and* ***(I)*** *Significant decrease of mitosis in melatonin animals (P < 0.01). (arrows indicate the positive expression). Bar= 50 µm.*

The quantification of this reduction in PCNA immunostaining was assessed by calculating the PCNA index. This analysis revealed that the PCNA protein expression was significantly

decreased in tumors sections of melatonin-pretreated animals (33%) compared to the control group (75%) at ($P < 0.005$) (Table 1). This result was further supported by the observation during the histopathological analysis which showed that the number of mitosis per millimeter was markedly decreased in melatonin treated group compared with non-treated mice ($P < 0.005$). Collectively, these data demonstrate that melatonin treatment impairs melanoma cell proliferation and supports our pervious result on tumor growth inhibition by melatonin.

Group	Shape of cells	Pigmented cells	Degeneration & necrosis	Angiogenesis	Mitotic index	PCNA index (%)
Control	Spindle	Very few	+	15.20±1.65	7.36±0.51	75±1.1
Melatonin treated	Epithelioid	+++	+++	5.86±1.65	2.83±0.29**	33±1.6**

Table 1: Summary of the histopathological and immunohistochemical reaction in control and melatonin-pretreated mice.

*Values are expressed as means ± S.E.M. Significant differences were measured using one-way ANOVA (**P < 0.01 compared with control mice).*

3.3. Histopathology.

A semi-quantitative evaluation of the histopathological features was conducted to assess cell morphology, vascularity, melanin and degeneration and necrosis as illustrated in Table 1. Microscopically, the tumors of control animals exhibited hypercellurarity with pleomorphic cells mostly spindle-oval and round shaped neoplastic cells associated with a well-developed and established vascularization and scanty pigmented cells; in the melatonin-pre-treated animals, tumors consisted of epithelioid-shaped neoplastic cells surrounded by blood capillaries forming lobular patterns and with obvious extension of necrosis. Interestingly, most of the neoplastic cells were more differentiated and exhibited melanin expression. This finding argues that orally administrated melatonin treatment attenuates melanoma growth.

3.4. Assays of mitochondrial activities.

As shown in Figure 3A, the respiratory control ratio (RCR), (state 3/state 4), was not significantly decreased in melatonin-treated mice. Mitochondrial complex activities, complex I, III or IV did not show any activity differences (Figure. 3B).The influence of oral treatment with melatonin on calcium-induced mitochondrial PTP opening is illustrated in Figure 3C. Addition of calcium stimulated a release of accumulated Ca^{2+}; this phenomenon was reduced by cyclosporine A. The larger AUC and increased level of PTP opening in melatonin-treated mice indicate that the calcium retention capacity of tumor cells in non-treated group was significantly lower than in the melatonin-treated mice. In melatonin-pre-treated animals, the level of mitochondrial nitrites was significantly higher compared with the control tumors (Figure 3D). The SOD activity did not exhibit any significant difference between control and treated mice (Fig 3E).

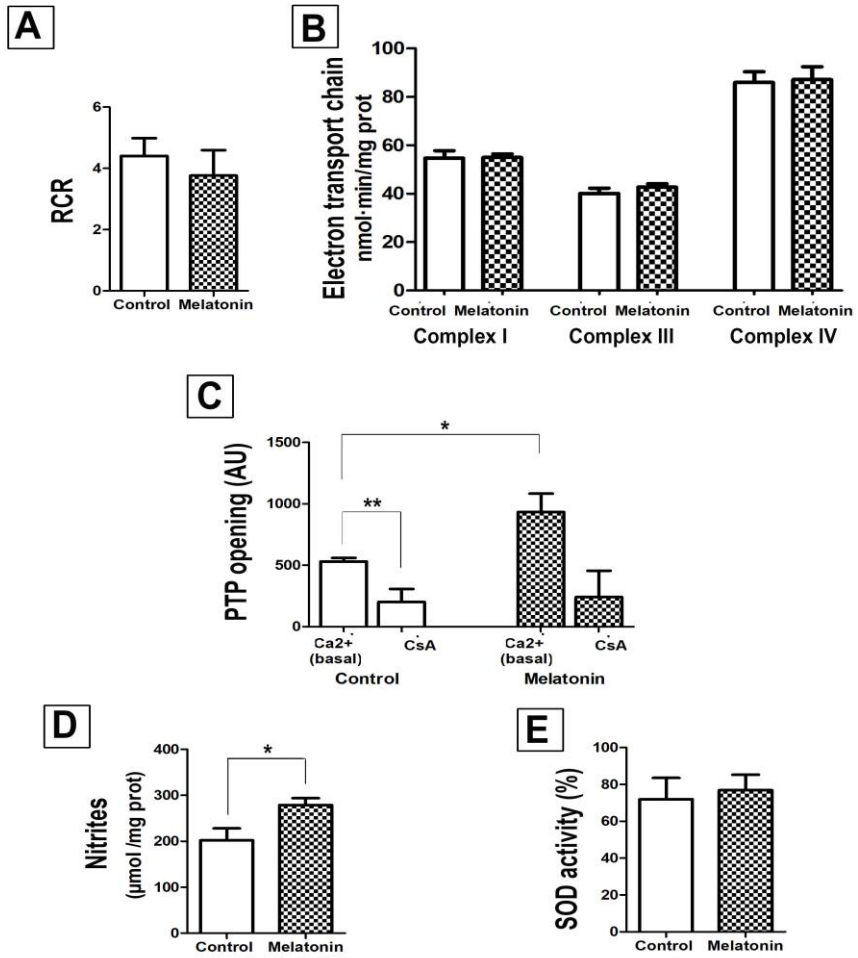

Fig. 3. Effects of melatonin on mitochondrial activities.

(A) *Respiratory parameters of mitochondria isolated from tumors of both, control (untreated) and melatonin-treated mice. Values are the means ± S.E.M.* ***(B)*** *Activities of the electron transport chain (ETC) complexes I, III, and IV in tumor mitochondria isolated from control (untreated) and melatonin-treated mice. Values are the means ± S.E.M.* ***(C)*** *Calcium-induced permeability transition pore (PTP) opening of mitochondria isolated from control (untreated) and melatonin-treated tumors. Values are the means ± S.E.M. Melatonin-treated animals exhibited a significant increase in PTP activation compared to control animals ($P < 0.05$). Addition of cyclosporine caused inhibition of PTP opening ($P < 0.005$).* ***(D)*** *Nitrate concentration in mitochondria isolated from control (untreated) and melatonin-treated tumors. Melatonin-treated mice had increased nitrite levels ($P < 0.05$). Values are the means ± S.E.M.* ***(E)*** *Superoxide dismutase (SOD) activity in mitochondria isolated from control (untreated) and melatonin-treated tumors. Values are the means ± S.E.M.*

3.5. MAPK signaling pathway is inhibited in melatonin-treated tumors.

Melatonin possesses anti-invasive/anti-metastatic actions in breast cancer cell lines that involve p38 MAPK signaling pathway inhibition and repression of epithelial-mesenchymal transition (EMT) (42). Also, MAPK signaling pathway is activated during malignant transformation of melanocytes and progression since the introduction of a constitutively active MAPKK in immortalized melanocytes led to tumorogenesis and increased expression of pro-angiogenic factors (VEGF) and pro-invasiveness molecules such as MMPs (21). Therefore, to test whether the melatonin negative effect on melanoma tumor growth was driven by inhibiting

MAPK signaling pathway, we investigated its status through phosphorylated ERK1 and 2 expression analysis by immunohistochemistry in melatonin-treated tumors and controls. The results revealed an abundant cytoplasmic with some nuclear staining in control groups, while the melatonin-treated tumors demonstrated rare expression although the endothelial lining of blood capillaries were positively immunostained (Fig 2G and H). The quantitative data of ERK1 and 2 provide a strong argument that MAPK signaling pathway is inhibited in melatonin-treated tumors.

4. DISCUSSION

Numerous *in vitro* studies have been conducted on the oncostatic effect of melatonin on breast, prostate and other types of cancers (43-46). Melatonin exerts both direct anticancer, antiproliferative, proapototic, differentiating, and indirect effect through free radicals scavenging and immunomodulatory actions (47). It has been proposed that the anti-proliferative effect of melatonin was exerted by increasing the expression of the tumour-suppressor gene p53 (48-49). Melatonin also has been shown to activate the apoptosis cascade through enhancing flavone-induced apoptosis in human cancer cells by increasing the level of glycolytic end products (50). In melanoma, nocturnal administration of melatonin prevents the circadian rhythmicity impairment associated with tumor progression in C57BL/6 mice (26). This inbred mouse strain has a point mutation in the AANAT gene, which may result in absence or impairment of the melatonin rhythm (51). However, in these mice serotonin is acetylated by an alternative enzyme (NAT) that is expressed peripherally, providing rationale for melatonin production in these mice (52-53).

To best of our knowledge, there is only one study which reported the absence of any preventive effect of non-aggressive, orally administered melatonin on melanoma tumor growth *in vivo* (28). We report here for the first time that orally administrated melatonin has an inhibitory effect B16-F10 melanoma tumor cell growth *in vivo* as illustrated by the significant tumor size on days 22, 25 and 33 in melatonin treated animals compared to the control mice. However, as it was previously reported that the slopes of the growth, once tumor is detectable in melatonin-pretreated animals, are similar. Moreover, melatonin pretreatment caused a latency period of almost two weeks in the appearance of measurable tumors. Our finding is in line with other previous reports on the anticancer effect of melatonin which appeared also to be heavily weighted toward suppression of breast cancer cell proliferation and enlargement (54). The physiologic concentrations of melatonin, which are similar to serum values in humans, significantly suppress the proliferation of human breast cancer cells *in vitro* (54).

In support our finding that orally administrated melatonin treatment impairs melanoma cells proliferation *in vivo*, we studied the PCNA protein expression in the tumors from control and melatonin-pretreated animals by immunostaining. This revealed a significant decrease of PCNA LI by melatonin treatment which indicates a reduction in cell proliferation of malignant melanoma cells growing in the subcutaneous tissue. This result not only confirms the previous observation about the inhibitory effect of orally administered melatonin on melanoma cells growth *in vivo* but provides additional evidence related the recently suggested key role of PCNA in the metastasis and invasive processes of melanoma (41).

Histopathological results of the tumors melatonin-treated animals showed the presence of phenotypic difference with the tumor cells having a epithelioid appearance, in addition to the presence of pigmented cells. This finding is likely attributable to tumor heterogeneity as pigmented cells are more resistant than undifferentiated ones as demonstrated by a recent study which clearly showed that cells containing melanin were less capable of spreading than cells without the pigment (55). Also, it has been reported that melatonin regulates melanin pigmentation in mammalian skin (56-57). Collectively, these data shed new light on the role

of melatonin pretreatment in enhancing the return of malignant cell to a more differentiated state expressing melanin so to reduce the aggressive phenotype.

It has been reported that melatonin induces cell death through two pathways; extrinsic or death receptor and the intrinsic or mitochondrial apoptosis. During mitochondrial-dependent cell death, ATP depletion, membrane rupture and activation of pro-apoptotic factors are produced, finally resulting in cell death (58-62).

PTP alterations are related with melatonin-mediated mitochondrial oxidative stress and increased mitochondrial nitrite level that might induce tumor cell toxicity. Moreover, the increased oxidative stress could also result in a reduction in tumor cells proliferation. In melanoma cell line (B16 melanoma 4A5), the lower melatonin concentration reported, reduced proliferation, while the cytotoxicity was observed exclusively at high concentrations of the indole associated with increased ROS levels, thus reducing the viability melanoma cell line (6). However, melatonin did not influence some mitochondrial parameters such as ETC complexes or SOD activity, which may indicate an irreversible mitochondrial damage.

The mitogen-activated protein kinase (MAPK) signalling pathway has a key role in regulating the growth and survival of a wide range of tumors (63) including melanoma (20-21. Also, MAPK signalling pathway is considered one of the most important therapeutic targets for the prevention and treatment of melanoma (64). Interestingly, we found that melatonin decreased phosphorylated ERK1 and 2 expressions in melanoma cancer cells in comparison with non-treated animals suggesting that the MAPK signaling pathway is inhibited in melatonin-treated tumors and may be responsible for melanoma cell growth delay.

On the basis of reduced mitotic figures, presence of pigmented cells, increased NO inducing cytotoxicity, and decreased MAPK signalling pathway activity involved in cell growth, associated with a lower PCNA index. The results of the current study suggest a possible chemopreventive role of orally administrated melatonin against induced malignant melanoma in mice.

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AUTHORSHIP

A.A. Study design, analysis and interpretation, drafting of manuscript critical revision of the manuscript and approval of the article. K.B. Acquisition of including respirometry, histology preparation, analysis of morphology, drafting, critical revision and approval of manuscript. M.N.A. Mitochondria preparation, respirometry and enzyme activity determinations, drafting of manuscript, critical revision of the manuscript and approval of the article. W.A. Pathological and immunohistochemical study and critical revision and approval of manuscript. L.Z. Penultimate drafting of manuscript critical revision of the manuscript and approval of the article. J.M.E. Statistical analysis. Acquisition of including tumor growth and survival, critical revision and approval of manuscript. R.J.R. Last drafting of manuscript, critical revision and approval of manuscript.

CONFLICT INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Slominski A WJ, Carlson AJ, Matsuoka LY, Balch CM, Mihm MC. (2001) Malignant melanoma: an update. *Arch. Pathol. Lab. Med.* **125**: 1295-306.
2. Setlow RB, Woodhead AD. (1994) Temporal changes in the incidence of malignant melanoma: Explanation from action spectra. *Mutat. Res.* **307**: 365-374.
3. Rigel DS. (2010) Epidemiology of melanoma. *Semin Cutan Med Surg*. **29**:204-209.
4. Grabacka M, Placha W, Plonka PM, Pajak S, Urbanska K, Laidler P, *et al*. (2004) Inhibition of melanoma metastases by fenofibrate. *Arch. Dermatol. Res*. **296**: 54-58.
5. Slominski A, Carlson AJ. (2014) Melanoma resistance: a bright future for academicians and a challenge for patient advocates. *Mayo Clin. Proc.* **89**: 429-433.
6. Bonmati-Carrion MA, Alvarez-Sanchez N, Hardeland R, Madrid JA, Rol MA. (2013) A comparison of B16 melanoma cells and 3T3 fibroblasts concerning cell viability and ROS production in the presence of melatonin, tested over a wide range of concentrations. *Int. J. Mol. Sci*. **14**: 3901-3920.
7. Acuña-Castroviejo D, Escames J, Venegas, C, Díaz-Casado ME, Lima-Cabello E, López LC, *et al*. (2014) Extrapineal melatonin: sources, regulation and potential functions. *Cell Mol. Life Sci*. **71**: 2997-3025.
8. Mocayar-Marón FJ, Ferder L, Reiter RJ, Manucha W. (2020) Daily and seasonal mitochondrial protection: Unraveling common possible mechanisms involving vitamin D and melatonin. *J. Steroid. Biochem. Mol. Biol.* **199**: e105595.
9. Hardeland R, Madrid JA, Tan DX, Reiter RJ. (2012) Melatonin, the circadian multioscillator system and health: the need for detailed analyses of peripheral melatonin signaling. *J. Pineal Res*. **52**: 139-166.
10. Tan DX, Chen L, Poeggeler B, Manchester L, Reiter RJ. (1993) Melatonin: a potent, endogenous hydroxyl radical scavenger. *Endocr J*. **1**: 57-60.
11. Agil A, Reiter RJ, Jimenez-Aranda A, Iban-Arias R, Navarro-Alarcon M, Marchal JA, *et al*. (2013) Melatonin ameliorates low-grade inflammation and oxidative stress in young Zucker diabetic fatty rats. *J. Pineal Res*. **54**:381-388.
12. Miller SC, Pandi PS, Esquifino AI, Cardinali DP, Maestroni GJ. (2006) The role of melatonin in immuno-enhancement: potential application in cancer. *Int. J. Exp. Pathol.* **87**: 81-87.
13. Srinivasan V, Spence DW, Trakht I, Pandi-Perumal SR, Cardinali DP, Maestroni GJ. (2008) Immunomodulation by melatonin: its significance for seasonally occurring diseases. *Neuroimmunomodulation* **15**: 93-101.
14. Reiter RJ, Tan DX, Poeggeler B, Menendez-Pelaez A, Chen LD, Saarela S. (1994) Melatonin as a free radical scavenger: implications for aging and age-related diseasesa. *Ann. NY. Acad. Sci.***719**: 1-12.
15. Gonzalez R, Sanchez A, Ferguson J, Balmer C, Daniel C, Cohn A, *et al*. (1991) Melatonin therapy of advanced human malignant melanoma. *Melanoma Res*. **1**: 237-244.
16. Slominski A, Pruski D. (1993) Melatonin inhibits proliferation and melanogenesis in rodent melanoma cells. *Exp. Cell Res.* **206**: 189-194.
17. Panzer A, Viljoen M. (997) The validity of melatonin as an oncostatic agent. *J. Pineal Res*. **22**: 184-202.
18. Lu KH, Lin RC, Yang JS, Yang WE, Reiter RJ, Yang SF. (2019) Molecular and cellular mechanisms of melatonin in osteosarcoma. *Cells* **8**: e1618.

19. Reiter RJ, Rosales-Corral SA, Tan DX, Acuna -Castroviejo D, Quin D, Yang SF, Xu K. (2019) Melatonin as full service antitumor agent: inhibitionof of inititantion, progression and metastasis. *Int. J. Mol. Sci.* **18**: E843.
20. Cohen C, Zavala-Pompa A, Sequeira JH, Shoji M, Sexton DG, Cotsonis G, *et al*. (2002) Mitogen-actived protein kinase activation is an early event in melanoma progression. *Clin. Cancer Res*. **8**: 3728-3733.
21. Govindarajan B, Bai X, Cohen C, Zhong H, Kilroy S, Louis G, *et al*. (2003) Malignant transformation of melanocytes to melanoma by constitutive activation of mitogen-activated protein kinase kinase (MAPKK) signaling. *J. Biol. Chem*. **278**: 9790-9795.
22. Wen SY, Cheng SY, Ng SC, Aneja R, Chen CJ, Huang CY, Kuo WW. (2019) Roles of p38α and p38β mitogen‐activated protein kinase isoforms in human malignant melanoma A375 cells. *Int. J. Mol. Med.* **44**: 2123-2132.
23. Cos S, Garcia-Bolado A, Sanchez-Barcelo E. (2001) Direct antiproliferative effects of melatonin on two metastatic cell sublines of mouse melanoma (B16BL6 and PG19). *Melanoma Res*. **11**:197-201.
24. Kadekaro AL, Andrade LN, Floeter-Winter LM, Rollag MD, Virador V, Vieira W, et al. (2004) MT-1 melatonin receptor expression increases the antiproliferative effect of melatonin on S-91 murine melanoma cells. *J. Pineal Res*. **36**:204-211.
25. Fischer T, Zmijewski M, Zbytek B, Sweatman T, Slominski R, Wortsman J, et al. (2006) Oncostatic effects of the indole melatonin and expression of its cytosolic and nuclear receptors in cultured human melanoma cell lines. *Int. J. Oncol*. **29**: 665-672.
26. Otalora BB, Madrid JA, Alvarez N, Vicente V, Rol MA. (2008) Effects of exogenous melatonin and circadian synchronization on tumor progression in melanoma-bearing C57BL6 mice. *J. Pineal Res*. **44**: 307-315.
27. Kleszczynski K, Kim TK, Bilska B, Sarna M, Mokrzynski K, Stegemann A, *et al.* (2019) Melatonin exerts oncostatic capacity and decreases melanogenesis in human MNT-1 melanoma cells. *J. Pineal Res.***67**: e12610.
28. Alvarez-Artime A, Cernuda-Cernuda R, Francisco-Artime-Naveda, Cepas V, Gonzalez-Menendez P, Fernadez-Vega S, Quiros-Gonzalez I, Sainz RM, Mayo JC. (2020) Melatonin-induced cytoskeleton reorganization leads to inhibition of melanoma cancer cell proliferation. *Int. J. Mol. Sci***. 21**: 1-20.
29. Prados J, Melguizo C, Ortiz R, Boulaiz H, Carrillo E, Segura A, Rodríguez-Herva JJ, Ramos JL, Aránega A. (2010) Regression of established subcutaneous B16-F10 murine melanoma tumors after gef gene therapy associated with the mitochondrial apoptotic pathway. *Exp. Dermatol.***19**: 363-371.
30. Gnaiger E, Mendez G, Hand SC. (2000) High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. *Proc. Natl. Acad. Sci. USA* **97**:11080-85.
31. Lopez A, Garcia JA, Escames G, Venegas C, Ortiz F, Lopez LC, *et al.* (2009) Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. *J. Pineal Res*. **46**: 188-198.
32. Ziegler DM, Doeg K. (1962) Studies on the electron transport system. XLIII. The isolation of a succinic-coenzyme Q reductase from beef heart mitochondria. *Arch. Biochem. Biophys*. **97**: 41-50.
33. Rieske J. (1967) Preparation and properties of reduced coenzyme Q-cytochrome c reductase (complex III of the respiratory chain). *Method. Enzymol*. **10**: 239-245.
34. Jimenez-Aranda A, Fernandez-Vazquez G, Campos D, Tassi M, Velasco-Perez L, Tan DX, *et al*. (2013) Melatonin induces browning of inguinal white adipose tissue in Zucker diabetic fatty rats. *J. Pineal Res*. **55**: 416-423.

35. Stuehr DJ, Nathan CF. (1989) Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med*. **169**: 1543-1555.
36. Morales AI, Detaille D, Prieto M, Puente A, Briones E, Arevalo M, *et al*. (2010) Metformin prevents experimental gentamicin-induced nephropathy by a mitochondria-dependent pathway. *Kidney Int*. **77**: 861-869.
37. Burton AL, Egger ME, Gilbert JE, Stromberg AJ, Hagendoorn L, Martin RC, *et al*. (2012) Assessment of mitotic rate reporting in melanoma. *Am. J. Surg*. **204**: 969-974; discussion 974-975.
38. Lawson DA, Kessenbrock K, Davis RT, Pervolarakis N, Werb Z. **(**2018) Tumour heterogeneity and metastasis at single-cell resolution. *Nat. Cell Biol*. **20**: 1349-1360.
39. Bravo R, Frank R, Blundell PA, Macdonald-Bravo H. (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. *Nature* **326**: 515-517.
40. Strzalza W, Ziemienowick A. (2011) Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. *Ann. Bot.* **107**: 1127-1140.
41. Chen J, Wu F, Shi Y, Yang D, Xu M, Lai Y, Liu Y. (2019) Identification of key candidate genes involved in melanoma metastasis. *Mol. Med. Rep*. **20**: 903-914.
42. Hill SM, Belancio VP, Dauchy RT, Xiang S, Brimer S, Mao L, *et al*. (2015) Melatonin: an inhibitor of breast cancer. *Endocr. Relat. Cancer* **22**: R183-204.
43. Watanabe M, Kobayashi Y, Takahashi N, Kiguchi K, Ishizuka B. (2008) Expression of melatonin receptor (MT1) and interaction between melatonin and estrogen in endometrial cancer cell line. *J. Obste.t Gynaecol. Res*. **34**: 567-573.
44. Rodriguez-Garcia A, Mayo JC, Hevia D, Quiros-Gonzalez I, Navarro M, Sainz RM. (2012) Phenotypic changes caused by melatonin increased sensitivity of prostate cancer cells to cytokine-induced apoptosis. *J. Pineal Res*. **54**: 33-45.
45. Proietti S, Cucina A, Reiter RJ, Bizzarri M. (2013) Molecular mechanisms of melatonin's inhibitory actions on breast cancers. *Cell Mol. Life Sci*. **70**: 2139-2157.
46. Lu KH, Lin RC, Hsieh YH, Su SC, Reiter RJ, Yang SF. (2020) New insights into antimetastatic signaling pathways of melatonin in skeletomuscular sarcoma of childhood and adolescence. *Cancer Metastasis Rev*. **39**: 303-320.
47. Di Bella G, Mascia F, Gualano L, Di Bella L. (2013) Melatonin anticancer effects: review. *Int. J. Mo.l Sci*. **14**: 2410-2430.
48. Mediavilla MD, Cos S, Sanchez-Barcelo EJ. (1999) Melatonin increases p53 and p21WAF1 expression in MCF-7 human breast cancer cells in vitro. *Life Sci*. **65**:415-420.
49. Stevens RG, Rea MS. (2001) Light in the built environment: potential role of circadian disruption in endocrine disruption and breast cancer. *Cancer Causes Control* **12**: 279-287.
50. Wenzel U, Nickel A, Daniel H. (2005) Melatonin potentiates flavone-induced apoptosis in human colon cancer cells by increasing the level of glycolytic end products. *Int. J. Cancer*. **116**: 236-242.
51. Roseboom PH, Namboodiri MA, Zimonjic DB, Popescu NC, Rodriguez IR, Gastel JA, *et al.* (1998) Natural melatonin 'knockdown' in C57BL/6J mice: rare mechanism truncates serotonin N-acetyltransferase. *Brain Res. Mol. Brain Res*. **63**: 189-197.
52. Slominski A, Pisarchik A, Semak I, Sweatman T, Wortsman J. (2003) Characterization of the serotoninergic system in the C57BL/6 mouse skin. *Eur. J. Biochem.* **270:** 3335-3344.
53. Slominski A, Wortsman J, Tobin DJ. (2005) The cutaneous serotoninergic /melatoninergic system: securing a place under the sun. *FASEB J.* **19**: 176-194.
54. Hill SM, Frasch T, Xiang S, Yuan L, Duplessis T, Mao L. (2009) Molecular mechanisms of melatonin anticancer effects. *Integr. Cancer Ther*. **8**: 337-346.
55. Sarna M, Krzykawska-Serda M, Jakubowska M, Zadlo A, Urbanska K. (2019) Melanin presence inhibits melanoma cell spread in mice in a unique mechanical fashion. *Sci. Rep*. **9**: 9280.

56. Slominski A, Pisarchik A, Wortsman J. (2004a) Expression of genes coding melatonin and serotonin receptors in rodent skin. *Biochim. Biophys. Acta* **1680**: 67-70.
57. Slominski A, Tobin DJ, Shibahara S, Wortsman J. (2004b) Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol. Rev*. **84**: 1155-1228.
58. Leo S, Bianchi K, Brini M, Rizzuto R. (2005) Mitochondrial calcium signalling in cell death. *FEBS J*. **272**:4013-4022.
59. Bernardi P, Krauskopf A, Basso E, Petronilli V, Blachly-Dyson E, Di Lisa F, *et al*. (2006) The mitochondrial permeability transition from in vitro artifact to disease target. *FEBS J*. **273**: 2077-2099.
60. Hajnoczky G, Csordas G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S, *et al*. (2006) Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. *Cell Calcium* **40**: 553-560.
61. Giorgi C, Romagnoli A, Pinton P, Rizzuto R. (2008) Ca2+ signaling, mitochondria and cell death. *Curr. Mol. Med*. **8**: 119-130.
62. Orrenius S, Nicotera P, Zhivotovsky B. (2011) Cell death mechanisms and their implications in toxicology. *Toxicol. Sci*. **119**: 3-19.
63. Johnson GL, Lapadat R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298**: 1911-1912.
64. Sebolt-Leopold JS, Herrera R. (2004) Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat. Rev. Cancer*. **4**: 937-947.

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