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Effect of Melatonin on Human Keratinocytes and Fibroblasts Subjected to UVA and UVB Radiation *In Vitro*

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Abstract. *Skin represents one of the extrapineal sites of melatonin (Mel) synthesis. In the skin Mel plays, for example, the role of an antioxidant which scavenges and inactivates free radicals arising due to UV irradiation. Although the protective effect of Mel on skin and cells irradiated mainly with UVB has been documented, to date no comparison has been made for the effects of Mel on cells exposed to UVA. Our study aimed at evaluating the effect of Mel (0, 10^{-3}, 10^{-6} or 10^{-9} M) added to culture medium 30 minutes before exposure of keratinocytes and fibroblasts to irradiation with UVA (15 J/cm^2) and UVB (30 mJ/cm^2, 60 mJ/cm^2). Viability of the cells was evaluated using sulphorhodamine (SRB) colorimetric test. Mel at 10^{-3} M increased the number of surviving keratinocytes and at 10^{-6} M increased the number of surviving fibroblasts exposed to UVB (30 mJ/cm^2, 60 mJ/cm^2) as compared to cells exposed only to radiation. In addition, 10^{-6} M protected keratinocytes exposed to the dose of 30 mJ/cm^2. Mel at 10^{-3} M exerted a protective effect on both types of cells irradiated with UVA (15 J/cm^2). As documented by our studies, Mel protects skin cells from the action of UVA and UVB. The protective effect of different Mel concentrations might result from variable expression of melatonin receptors.*

UV radiation (UVR) which reaches the Earth's surface and acts on human skin comprises 92% UVA spectrum and 8% UVB spectrum (1). Under the effect of UVR, reactive oxygen species (ROS) and reactive nitrogen species (RNS) rapidly appear in skin cells, damaging proteins, lipids and nucleic acids (2). In studies on the effect of various UVR types on the skin, UVB was noted to be absorbed mainly by epidermis, in which it exerts damaging effects on *e.g.* keratinocyte DNA, inducing apoptosis of the cells (3). UVB acts also on fibroblasts of the dermis, accelerating its aging (4). Fibroblasts are less resistant to UVB than are keratinocytes because they contain a less efficient system for genome repair (5). The more resistant keratinocytes, on the other hand, due to such damage are more effectively removed from epidermis by apoptosis, which prevents their neoplastic transformation (5). Most UVA is absorbed by the dermis and is responsible for apoptosis of fibroblasts and skin aging (6). High doses of UVA to the epidermis may lead to apoptosis of keratinocytes (7). Both UVB and UVA promote the development of various forms of skin malignancies (8, 9).

A natural substance which prevents against UVR-induced damage and the oxidative stress evoked by it may be melatonin (Mel), the pineal hormone which is also produced in the skin (10). In keratinocytes and fibroblasts, expression of the enzymes participating in the synthesis of Mel (tryptophan hydroxylase, TPH; serotonin *N*-acetyl-transferase, NAT; hydroxyindole-*O*-methyltransferase, HIOMT) was shown, as well as expression of the melatonin receptor, MT1 (11, 12). Moreover, both types of cells express the other Mel receptors: the intracellular receptor (quinone oxidoreductase 2, MT3) and the nuclear receptor, RORα (retinoid-related orphan receptor α) (13). Under the effect of UVR, the process of Mel transformation to 2-hydroxy-melatonin and *N*-acetyl-*N*-formyl-5-metoxykinuramine (AFMK) becomes intensified in the skin (14). Mel and products of its metabolism form the melatoninergic antioxidative system which protects the skin from ROS and RNS, induced by UVR (14). In studies on the protective effect of Mel on UV-irradiated cells, Mel was found to attenuate expression of genes engaged in carcinogenesis and to inhibit ROS formation in leukocytes and in cornea cells (15-17). Mel also reduced apoptosis of HaCaT keratinocytes (immortalized keratinocytes) subjected to UVR action and apoptosis of human fibroblasts and leukaemia cells of the U937 cell line subjected to the action of UVB (18-20). In *in vivo* studies,

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Mel applied together with vitamins C and E on a patient's skin before irradiation with UVB prevented development of erythema. In turn, Mel administered intraperitoneally protected UVB-irradiated lens from lesions which initiate the development of a cataract (21, 22).

Taking these data into account and the low number of experiments which investigated the effect of Mel on survival of UV-irradiated human keratinocytes and fibroblasts, the aim of the present study was to determine the effect of Mel on these cells when exposed to the action of UVA and UVB.

Materials and Methods

Melatonin. Mel (Sigma-Aldrich Chemie, Steinheim, Germany) was dissolved in 96% ethanol to obtain a starting concentration of 100 mM. Subsequently, Mel was diluted in culture medium and added to cells at a concentration of 0, 10^{-3}, 10^{-6} and 10^{-9} M 30 minutes before exposure of the cells to UVA and UVB. In addition, the effect of 24-hour cell incubation with Mel concentrations of 10^{-3}–10^{-9} M was examined.

Cell cultures. A cell line of normal keratinocytes (normal human epidermal keratinocytes, NHEK; Cambrex Bio Science, Walkersville, MD, USA), originating from the skin of an adult individual, was cultured in Keratinocyte Basal Medium with addition of bovine pituitary extract, recombinant human epidermal growth factor (rhEGF), insulin, hydrocortisone, gentamycin sulphate and amphotericin B (Lonza, Walkersville, MD, USA). A cell line of human dermal fibroblasts (normal human dermal fibroblasts, NHDF; Cambrex Bio Science) was cultured in Fibroblast Basal Medium-2 with supplementation of 10% foetal bovine serum, insulin, r-human fibroblast growth factor (rhFGF), gentamycin sulphate and amphotericin B (Lonza). The cell cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. Forty-eight hours before UV irradiation, the cells were removed from culture flasks using 0.25% trypsin-EDTA solution (Sigma-Aldrich Chemie) and seeded at 1×10^4 cells/well in a 48-well plate.

Irradiation of cells. The cells were exposed to UVB radiation using an Ultraviolet Lamp (6 W; Cole-Parmer, Vernon Hills, IL, USA) delivering the 280-320 nm wavelength range, with maximum emission at 312 nm. The source of UVA radiation was a High-intensity Longwave Ultraviolet Lamp (100 W; Cole-Parmer) which emitted a wavelength range of 320-400 nm, with maximum emission at 365 nm. The energy of UVB and UVA emission were 0.5 mW/cm^2 and 21 mW/cm^2 at a distance of 15 cm and 5 cm, respectively, from the irradiated cells. The cells were exposed to the following doses of UVB: 10 mJ/cm^2, 30 mJ/cm^2 and 60 mJ/cm^2, or to doses of UVA: 5 J/cm^2, 15 J/cm^2, 30 J/cm^2 and 45 J/cm^2. For the studies on Mel, the doses of 30 mJ/cm^2 and 60 mJ/cm^2 were selected for UVB, since the higher dose approached the minimum UVB dose (70 mJ/cm^2) required to evoke erythema (3). In the same studies, the point of reference for UVA used for irradiation (15 J/cm^2) was the dose of 25 J/cm^2 which induced apoptosis of numerous fibroblasts forming the dermis-imitating construct (6). Before irradiation, the cells were incubated for 30 minutes in medium containing different concentrations of Mel: 0, 10^{-3}, 10^{-6} and 10^{-9} M. Following incubation, the cells were washed in phosphate-buffered saline (PBS) and irradiated in a small amount of fresh PBS. The control involved non-irradiated cells. All the procedures were executed under dimmed light. Following irradiation, the PBS was removed and culture medium was added. The number of cells was determined using the sulphorhodamine B (SRB) colorimetric test twenty-four hours after irradiation and expressed relative to non-irradiated cell control. All the experiments were performed in triplicates.

Cell survival test. The number of living cells was determined using the colorimetric SRB test evaluating the amount of SRB bound by living cells. Twenty-four hours after irradiation, the cells were fixed with 50% trichloracetic acid and subsequently stained with 0.4% solution of SRB in 1% acetic acid for 30 minutes. The unbound dye was removed by washing in 1% acetic acid and the cell protein-bound dye was extracted with 10 mM unbuffered Tris solution. The optical density (OD) of the solution was read in an Elx 800 microplate reader (Bio-Tek, Instruments Inc., Vinooski, VT, USA) at 562 nm (23). Control for the read-out included the medium alone subjected to the above procedure. All the reagents for the test were purchased from Sigma-Aldrich Chemie.

Statistical analysis. Significance of differences between individual groups of obtained results used the Kruskal-Wallis test, as a non-parametric equivalent of the analysis of variance, in which the value of $p<0.05$ was accepted as indicating statistical significance. All statistical analyses were conducted using Statistica 7.1 software (StatSoft, Krakow, Poland).

Results

Keratinocytes. Irradiation of keratinocytes with increasing doses of UVB (10 mJ/cm^2, 30 mJ/cm^2, 60 mJ/cm^2) resulted in decreasing numbers of viable cells as compared to non-irradiated cells, which translated to proportions as follows: 95% at 10 mJ/cm^2, 83% at 30 mJ/cm^2 and 77% at 60 mJ/cm^2 ($p<0.05$) (Figure 1). UVA also exerted a lethal effect on keratinocytes and negatively affected cell survival as compared to non-irradiated cells: 90% at 5 J/cm^2, 93% at 15 J/cm^2, 87% at 30 J/cm^2 and 75% at 45 J/cm^2 ($p<0.05$) (Figure 2). Incubation of keratinocytes with Mel at concentrations ranging from 10^{-3} to 10^{-9} M for 24 hours significantly increased the number of cells in culture by 30% to 35% at all Mel concentrations as compared to the control without Mel ($p<0.05$) (Figure 3). Mel added at 10^{-3} and 10^{-6} M 30 minutes before irradiation of keratinocytes with 30 mJ/cm^2 UVB increased the number of viable cells, which was comparable to the number of cells subjected to no UVB action. Moreover, the number of cells in cultures with these concentrations of Mel was significantly higher ($p<0.05$) than the cell number in cultures without Mel. The concentration of 10^{-9} M provided no protective effect (Figure 4). A distinct effect was obtained when the cells were exposed to the higher UVB dose of 60 mJ/cm^2. The concentration of 10^{-3} M proved to be more effective as it augmented the fraction of

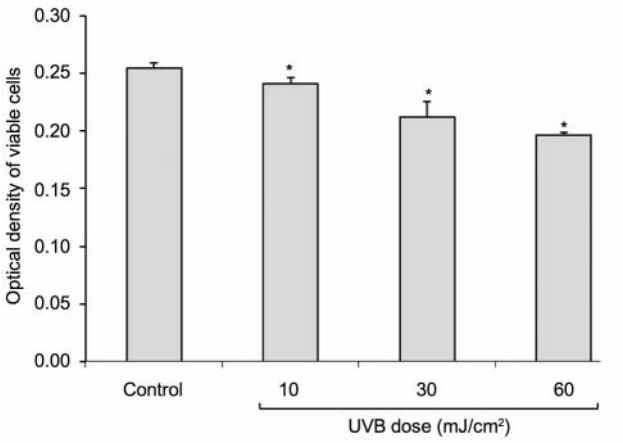

Figure 1. *Cell viability of UVB-irradiated keratinocytes. *p<0.05 vs. unirradiated cells (control).*

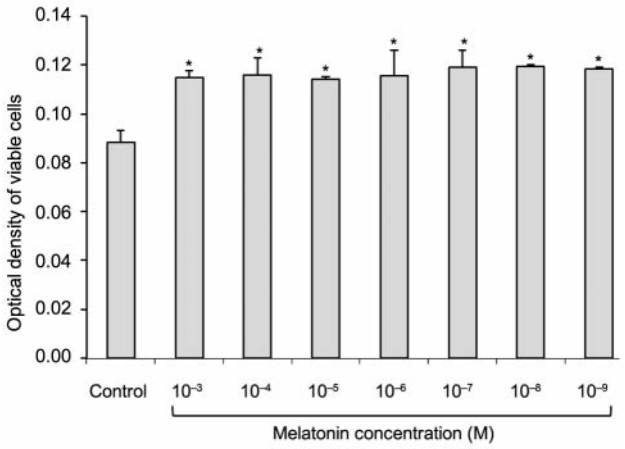

Figure 3. *Cell viability of keratinocytes incubated for 24 hours with Mel. *p<0.05 vs. cells without Mel (control).*

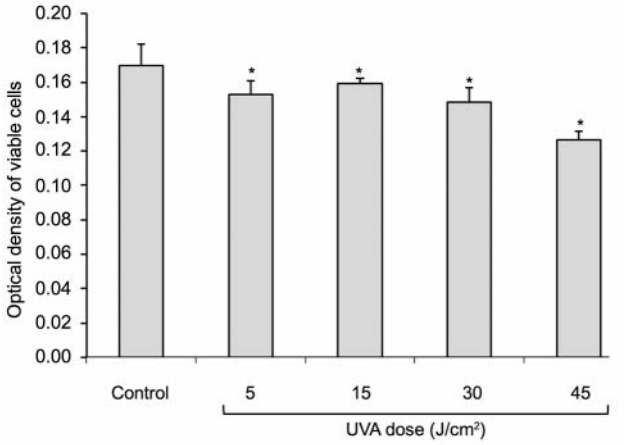

Figure 2. *Cell viability of UVA-irradiated keratinocytes. *p<0.05 vs. unirradiated cells (control).*

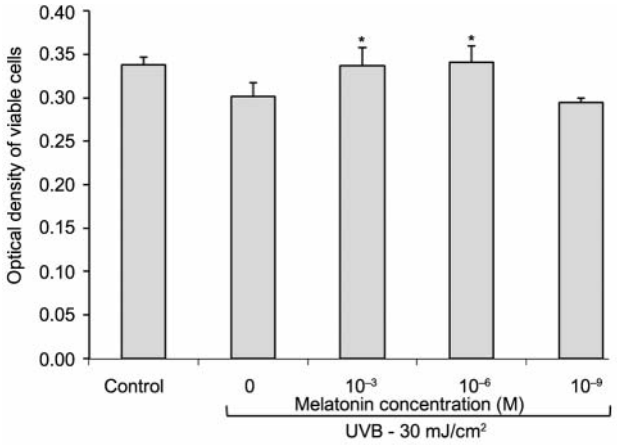

Figure 4. *Cell viability of keratinocytes preincubated for 30 minutes with Mel (0, 10^{-3}, 10^{-6} or 10^{-9} M) and irradiated with 30 mJ/cm² UVB. *p<0.05 vs. 0 M.*

viable cells to 92%, as compared to cultures not incubated with Mel (73%), or cultures exposed to the remaining Mel concentrations ($p<0.05$) (Figure 5). When UVA was used, only the highest Mel concentration (10^{-3} M) protected keratinocytes against irradiation with the dose of 15 J/cm^2, increasing the fraction of surviving cells to 91% as compared to cells not incubated with Mel (70%) ($p<0.05$) (Figure 6).

Fibroblasts. Irradiation of fibroblasts with increasing doses of UVB (10 mJ/cm^2, 30 mJ/cm^2, 60 mJ/cm^2) resulted in the decrease of cell numbers to 81% following a UVB dose of 10 mJ/cm^2, to 66% following 30 mJ/cm^2 and to 53% following 60 mJ/cm^2 ($p<0.05$) (Figure 7). Exposure to UVA resulted in a similar trend, although it was associated with different doses of radiation. The viable cells, as compared to unirradiated cells, comprised 91% following exposure to 5 J/cm^2, 84% after the dose of 15 J/cm^2, 76% after the dose of 30 J/cm^2 and 60% after the dose of 45 J/cm^2 ($p<0.05$) (Figure 8). Incubation of fibroblasts with Mel at concentrations ranging from 10^{-4} to 10^{-9} M for 24 hours increased the fraction of viable cells by 6% to 17% as compared to the control (cells without Mel) ($p<0.05$). The highest Mel concentration of 10^{-3} M proved to be toxic for fibroblasts and their number decreased to 60% of the control (Figure 9). Mel at 10^{-6} M added 30 minutes before exposure of fibroblasts to 30 mJ/cm^2 UVB significantly augmented the proportion of living cells (71%

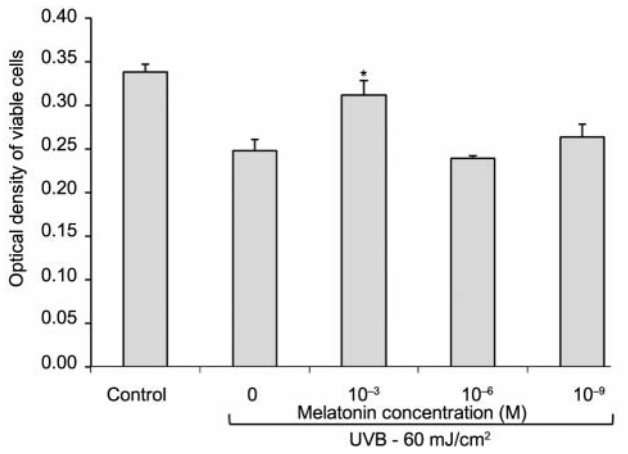

Figure 5. *Cell viability of keratinocytes preincubated for 30 minutes with Mel (0, 10^{-3}, 10^{-6} or 10^{-9} M) and irradiated with 60 mJ/cm² UVB. *p<0.05 vs. 0 M.*

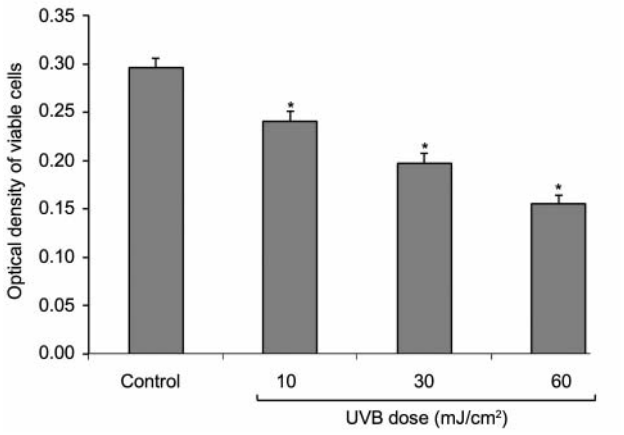

Figure 7. *Cell viability of fibroblasts irradiated with UVB. *p<0.05 vs. unirradiated cells (control).*

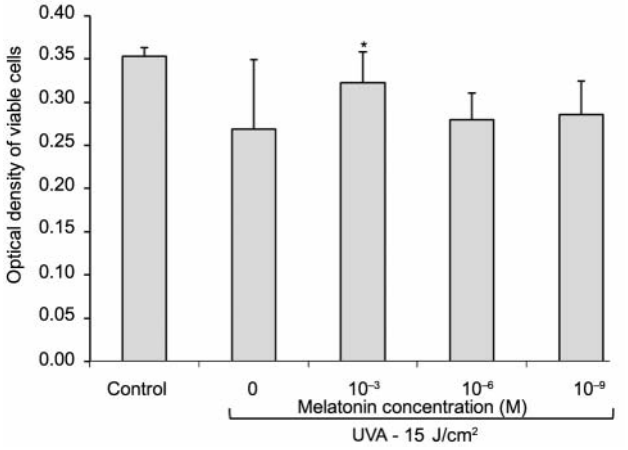

Figure 6. *Cell viability of keratinocytes preincubated for 30 minutes with Mel (0, 10^{-3}, 10^{-6} or 10^{-9} M) and irradiated with 15 J/cm² UVA. *p<0.05 vs. 0 M.*

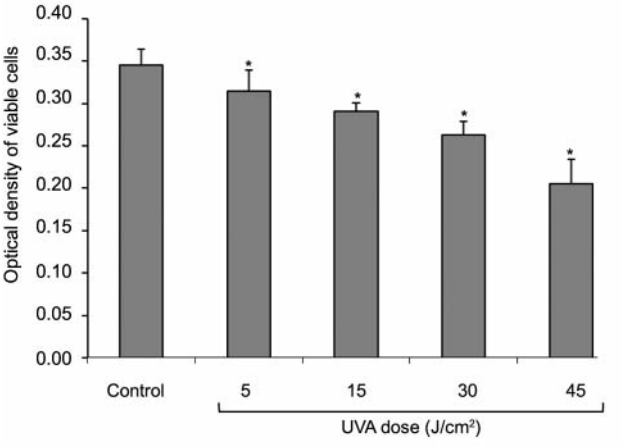

Figure 8. *Cell viability of fibroblasts irradiated with UVA. *p<0.05 vs. unirradiated cells (control).*

of viable cells), while cell cultures without Mel contained 66% of viable cells ($p<0.05$). No such effect was noted for Mel at concentrations of 10^{-3} or 10^{-9} M (Figure 10). Similarly, following irradiation of fibroblasts with the higher UVB dose of 60 mJ/cm², 10^{-6} M Mel exerted a protective effect (63% viable cells) as compared to cell cultures without Mel (55% viable cells) and cell cultures with the remaining Mel concentrations ($p<0.05$) (Figure 11). When the cells were exposed to UVA at a dose of 15 J/cm², only the highest Mel concentration (10^{-3} M) exerted a protective effect and increased the number of viable fibroblasts to 95% as compared to cell cultures without Mel (86%) ($p<0.05$). Mel at 10^{-6} and 10^{-9} M did not affect cell viability (Figure 12).

Discussion

Our earlier investigations have demonstrated that Mel on one hand decreases cardiotoxicity and nephrotoxicity of anthracyclines related to formation and activity of free oxygen species in normal tissues and, on the other, it intensifies their cytotoxic effect on neoplastic cells (24-26). The pronounced synthesis of Mel and expression of respective receptors in the skin, as well as its ability to scavenge ROS and RNS, make the hormone particularly interesting for dermatologists. Numerous *in vivo* and *in vitro* studies have demonstrated that Mel protects skin cells from the damaging effects of UVR (27). Since UVA and UVB radiation affect skin in a distinct manner, our study aimed at

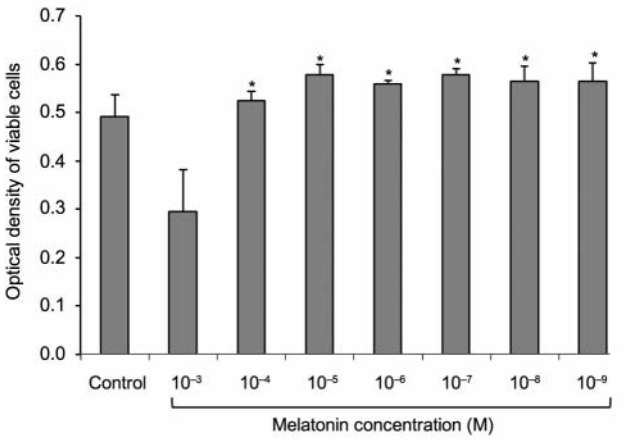

Figure 9. *Cell viability of fibroblasts incubated for 24 hours with Mel. *p<0.05 vs. cells without Mel (control).*

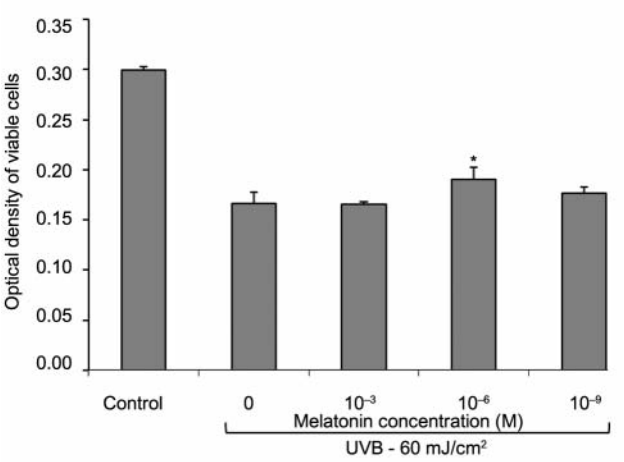

Figure 11. *Cell viability of fibroblasts preincubated for 30 minutes with Mel (0, 10^{-3}, 10^{-6} or 10^{-9} M) and irradiated with 60 mJ/cm^2 UVB. *p<0.05 vs. 0 M.*

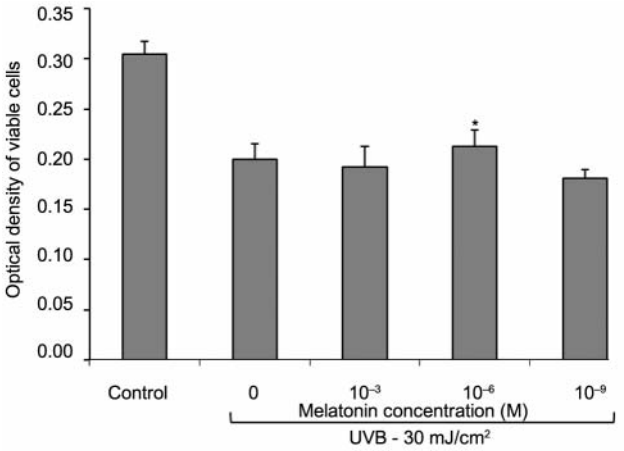

Figure 10. *Cell viability of fibroblasts preincubated for 30 minutes with Mel (0, 10^{-3}, 10^{-6} or 10^{-9} M) and irradiated with 30 mJ/cm^2 UVB. *p<0.05 vs. 0 M.*

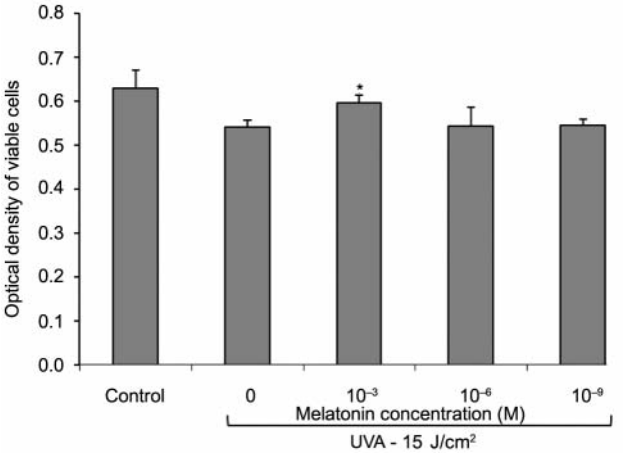

Figure 12. *Cell viability of fibroblasts preincubated for 30 minutes with Mel (0, 10^{-3}, 10^{-6} or 10^{-9} M) and irradiated with 15 J/cm^2 UVA. *p<0.05 vs. 0 M.*

evaluating the effect of the two types of radiation on cells originating from various skin layers: keratinocytes originating from epidermis and fibroblasts originating from dermis. It is worth stressing that the cells employed by us represent normal human skin cells and, therefore, the processes which take place in them may reflect the processes taking place *in vivo*.

Mel is endogenously synthesized in the skin and, therefore, we examined its effects at various concentrations on keratinocytes and fibroblasts *in vitro*. A 24-hour incubation of the two cell types with Mel increased their number as compared to cells subjected to no Mel activity. Only the highest concentration was proven to be cytotoxic to fibroblasts. Our observation finds confirmation in the data of Hipler *et al*. (28), where Mel stimulated proliferation of HaCaT keratinocytes. In another study, Mel was shown to stimulate proliferation of fibroblasts cultured in a serum-free medium (12).

Since *in vivo* Mel exhibits a short half-life of around 30 minutes in serum and it is rapidly turned over due to scavenging of ROS and RNS, its route of administration plays a significant role. In *in vivo* and *in vitro* experiments Mel applied on a patient's skin or added to cell culture for 15-30 minutes before irradiation with UVB prevented development of erythema and reduced the number of cells which underwent apoptosis. Administration of Mel following the exposure failed to protect the skin from erythema, or delayed the process of

apoptosis (20, 29, 30). In our studies, 30-minute incubation with Mel at 10^{-3} or 10^{-6} M increased the survival of keratinocytes subjected to UVB at the dose of 30 mJ/cm^2. The protective effect of the lower Mel concentration might have resulted from activation of MT1 receptor, the expression of which has been demonstrated in human keratinocytes. Following exposure to a higher dose of UVB (60 mJ/cm^2), only the highest Mel concentration (10^{-3} M) was able to protect keratinocytes. Similarly, in studies of Fischer *et al.* (18), Mel administered at a concentration of 10^{-3} or 10^{-4} M to HaCaT keratinocytes 30 minutes before irradiation with 50 mJ/cm^2 UVB significantly increased survival of cells as compared to cells exposed exclusively to radiation. No MT1 receptor expression takes place in HaCaT keratinocytes and perhaps for this reason the lower Mel concentrations exerted no effect on survival of the irradiated cells (18). The augmented survival of keratinocytes observed by us and others might have arisen from the effect of Mel-induced inhibition of pro-apoptotic protein and inhibition of gene expression linked to apoptosis and oxidative stress (31, 32).

Compared to keratinocytes, Mel proved to be less effective in the protection of fibroblasts irradiated with UVB doses of 30 mJ/cm^2 or 60 mJ/cm^2 since a Mel concentration of 10^{-6} M increased cell survival only slightly, as compared to non-irradiated cells. This relationship differs from results obtained by Ryoo *et al.* (19), where Mel added at the concentration of 10^{-7} or 10^{-9} M to fibroblasts 1 h before and, in addition, for 24 h after irradiation with 140 mJ/cm^2 significantly augmented cell survival as compared to cells exposed only to irradiation. The difference may have been related to differences in the setup of the experiment.

Our studies have, for the first time, demonstrated a protective effect of Mel on UVA-exposed human keratinocytes and fibroblasts. The effect of the highest Mel concentration (10^{-3} M) on survival of the two cell types may point to a similar mechanism of Mel action on different cells subjected to free radical stress. Similarly to UVB-irradiated keratinocytes and fibroblasts, it remains necessary to corroborate involvement of melatonin receptors in the process of protection from UVA since in a similar model of irradiation, MT1 and MT2 receptors were involved in protection by Mel of U937 leukaemia cells from UVB-induced apoptosis (20, 33). The low protective activity of Mel might be explained by its presence in only low amounts which pass across cell membranes and directly scavenge free radicals (34). Therefore, growing hopes are linked to application of liposome capsule-enclosed Mel (35). Hopefully, the increased intracellular concentration of Mel will increase its protective effects. In a similar manner in our experiments, we have demonstrated that only selected Mel concentrations in the culture medium may play a protective role against UVA and UVB effects. As already mentioned above, this may be linked to several variables, including receptor mechanism, different Mel administration, or variable ability of Mel to penetrate cell membranes. Nevertheless, beyond doubt, we have confirmed that Mel represents a substance which protects human keratinocytes and fibroblasts against effects of UVA and UVB in *in vitro* experiments. These results may be used in subsequent studies on Mel and in experiments on comprehensive application of Mel as a protection from UVR not only in its clinical aspects.

Acknowledgements

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