Melatonin and its metabolites ameliorate UVB-induced damages in human epidermal keratinocytes

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Abstract

We investigated the protective effects of melatonin and its metabolites: 6-hydroxymelatonin (6-OHM), N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), *N*-acetylserotonin (NAS), and 5-methoxytryptamine (5-MT) in human keratinocytes against a range of doses (25, 50, and 75 mJ/cm^2) of ultraviolet B (UVB) radiation. There was significant reduction in the generation of ROS (50–60%) when UVB-exposed keratinocytes were treated with melatonin or its derivatives. Similarly melatonin and its metabolites reduced the nitrite and hydrogen peroxide levels that were induced by UVB as early as 30 min after the exposure. Moreover, melatonin and its metabolites enhanced levels of reduced glutathione in keratinocytes within 1 h after UVB exposure in comparison to control cells. Using proliferation assay, we observed a dose-dependent increase in viability of UVB-irradiated keratinocytes that were treated with melatonin or its derivatives after 48 h. Using the dot-blot technique and immunofluorescent staining we also observed that melatonin and its metabolites enhanced the DNA repair capacity of UVB-induced 6-4PP or CPD generation in human keratinocytes. Additional evidence for induction of DNA repair in cells exposed to UVB and treated with the indole compounds was shown using the Comet assay. Finally, melatonin and its metabolites further enhanced expression of p53 phosphorylated at Ser-15 but not at Ser-46 or its non-phosphorylated form. In conclusion, melatonin, its precursor NAS, and its metabolites 6-OHM, AFMK, 5-MT, which are endogenously produced in keratinocytes, protect these cells against UVB-induced oxidative stress and DNA damage.

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Author Contributions

Keywords

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Introduction

Ultraviolet radiation (UVR) spectrum of solar light represents the major skin stressor. UVR-related damage to the skin and epidermal keratinocytes are tightly connected with the production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and nitric oxide (NO), which can cause oxidative damage and further reduction of the important antioxidant glutathione (GSH) and inhibition of DNA repair, in addition to genotoxic and mutagenic effects caused by these [1]. The mutagenic and genotoxic effects of UVR are predominantly related to absorption of UVB by DNA which acts as chromophores for wavelengths in the range of 290–320 nm [2]. Exposure to UVR causes DNA damage in form of primarily cyclobutane pyrimidine dimers (CPD) and to lower degree of pyrimidine photoproducts (6-4)PPs [3]. CPDs are DNA lesions produced in human skin exposed to either UVB or UVA [4], albeit lower levels for UVA exposure are required [3].

Melatonin (N-acetyl-5-methoxytryptamine) and its metabolites have multiple functions. They regulate circadian rhythms, biological responses, act as hormone, neurotransmitters, antiinflammatory agents, and as antioxidants [5–13]. These effects are secondary to activation of membrane bound receptors or are receptors independent [14, 15]. Melatonin's main sites of production are the pineal gland and retina, where serotonin is acetylated to N-acetylserotonin (NAS), which is further methylated, to melatonin [10, 16, 17]. Melatonin is also produced in peripheral organs including the skin [12, 13, 18]. It is well documented that mammalian skin has the capability of transforming L-tryptophan to serotonin that is further metabolised in this organ to melatonin [19–21]. Also, of note is that significant amounts of NAS accumulate in the skin as the main intermediate of the melatonin pathway [12, 20–23]. Melatonin is metabolised through the indolic and kynuric pathways resulting in the production of 6-hydroxymelatonin (6-OHM), cyclic 3-hydroxymelatoni, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), and 5-methoxytryptamine (5-MT) as main metabolites along with several additional products [reviewed in [11, 13, 24, 25]]. The production of AFMK occurs via the kynuric pathway [11, 25], although it can also be generated non-enzymatically through the interaction of melatonin and H_2O_2 [26, 27] or through the photochemical transformation secondary to exposure to UVB [27, 28]. NAS, a precursor to melatonin, is also a product of its metabolism [29], during the process of demethylation [13, 30].

The main product of melatonin metabolism in human keratinocytes is 6-OHM the production of which is stimulated by UVB exposure [28, 31]. 5-MT is a product of melatonin deacetylation, which in addition to retina is also produced in rodent skin [19, 23] and human epidermal keratinocytes [28, 31]. UVB can both directly induce transformation of melatonin to 2-hydroxymelatonin and AFMK [28] or stimulate the endogenous

production of AFMK in human epidermal keratinocytes [28, 31]. Thus, human epidermal keratinocytes produce NAS, melatonin, AFMK and 5-MT [18, 20, 28, 31]

Melatonin is recognised as an important protective factor in many different cells [11, 32–35]. Melatonin and its metabolites scavenge both ROS/RNS through a free radical scavenging cascade [11, 27, 34, 36]. Beside their antioxidative properties [37], melatonin and its metabolites exhibit antiproliferative and prodifferentiation properties in human epidermal keratinocytes [31, 38, 39]. These effects have been described mainly in intact histocultured skin or in HaCaT immortalized human keratinocytes [28, 31, 39–42]. Although, the protective effects of melatonin and AFMK have been studied extensively, there is a paucity of data supporting the protective role of NAS and other melatonin derivatives including 6-OHM and 5-MT. Furthermore, information on protective effects of melatonin's metabolites on primary epidermal keratinocytes cultured in vitro is limited. Therefore, in the current study we investigated the antioxidative and protective effects of melatonin metabolites in comparison to melatonin in normal human epidermal keratinocytes exposed to a range of doses of UVB (25, 50, and 75 mJ/cm^2). Since melatonin reportedly prevents DNA damage in cancer cells [43], in the current study we tested the ability of melatonin and its metabolites to prevent DNA damage in skin cells. p53 expression increases with UVB exposure and it initiates growth arrest and damaged DNA has been detected [44]. Melatonin induces a p53-dependent DNA damage response [45] and p53 protein expression [46]. We hypothesised that the UVR-induced increase in p53 is further augmented by melatonin derivatives and that this effect likely contributes to enhanced repair of UVR-induced thymine dimers.

Material and Methods

Chemicals

Melatonin (*N*-acetyl-5-methoxytryptamine) (M5250), 6-hydroxymelatonin (3-(N-acetylaminoethyl)-6-hydroxy-5-methoxyindole) (H0627), *N*-acetyl-5-hydroxy-tryptamine (N-acetylserotonin, normelatonin) (A1824), 5-methoxy tryptamine (2-(5-methoxyindol-3-yl) ethylamine, 3-(2-aminoethyl)-5-methoxyindole, 5-methoxyindole-3-ethanamine, 5-MT, deacetylmelatonin) (286583) were purchased from Sigma-Aldrich (St. Louis, MO); and AFMK (N1-acetyl-N2-formyl-5-methoxykynuramine, N-[3-[2-(formylamino)-5-methoxyphenyl]-3-oxypropyl]-acetamide) (10005254) from Cayman Chemical Company (Ann Arbor, MI, USA). Chemicals were prepared and used according to the protocols described elsewhere [47]. Ethanol was used as a dilutent and a vehicle.

Cell culture

HaCaT keratinocytes were grown in Dulbecco's minimal essential media (DMEM) supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA) [38, 48]. Under the current experimental conditions charcoal-treated FBS was used (Atlanta Biologicals, Lawrenceville, GA, USA). Human epidermal keratinocytes (HEKn) are isolated from neonatal foreskin of African-American donors as previously described [49, 50]. Cells were grown in keratinocyte growth media (KGM) supplemented with keratinocyte growth factors (KGF) (Lonza Walkersville Inc, Walkersville, MD). Cells in their third

passage were treated with melatonin or its derivatives for 24 h before UVB exposure and for various times after UVB exposure. For UVB exposure the media were replaced with phosphate buffered saline (PBS).

UVB exposure

The UV irradiation was performed with a Biorad UV transluminator 2000 (Bio-RAD Laboratories, Hercules, CA, USA) as previously described [41, 51]. For the experiments, doses of 25, 50, 75, or 200 mJ/cm^2 were chosen. With the emittance of UVB by the UV-source of 4.01 mW/cm^2 these doses were achieved after 20, 40, 60, or 120 s of irradiation, respectively.

Cell proliferation assays

HEKn keratinocytes cells were plated in 96-well plates, 10,000 cells/well. After overnight incubation, melatonin and its metabolites, initially dissolved in ethanol, were added to the medium to achieve final concentrations ranging from 1 mM–0.1 pM, while corresponding ethanol concentrations served as a control. After 44 h of incubation with the compounds, 20 µl of MTS/PMS solution (Promega, Madison, WI) was added to the cells. Four hours later, absorbance was recorded at 490 nm using an ELISA plate reader. The number of viable cells was measured in six replicates.

Comet assay

Keratinocytes were plated in 12-well plates. Cells were treated with melatonin, or its metabolites at the concentration of 5×10^{-5} M, or ethanol (vehicle, dilution 5:1,000) for 24 h. Then the medium was replaced with 1xPBS and cells were exposed to UVB 200 mJ/cm^2. PBS was removed and replaced with fresh medium containing compounds and cells were further incubated for 3 h at 37°C. After detaching, cells were counted and used for the comet assay. The comet assay was performed following the manufacturer's protocol (Trevigen, Gaithersburg, MD) as previously described [52]. Cell suspensions at a density of 1×10^5/ml were combined with molten 1.2% low-melting-point agarose, diluted 1:10, placed onto two frosted slides precoated with 0.6% normal agarose, and incubated at 4°C for 30 min. Cells were further digested in lysis solution at 4°C for 60 minutes. DNA strand breaks were separated by electrophoresis in alkaline electrophoreses solution (200 mM NaOH, 1mM EDTA, pH>13) in horizontal gel electrophoresis slide tray (Comet-10, Thistle Scientific, UK). DNA breaks were exposed to alkaline unwinding for 20 min at room temperature after which electrophoresis was performed at 25 V for 1 h. Following neutralization in neutralizing buffer (0.4 M Tris, pH7), comets are visualized by ethidium bromide (Sigma-Aldrich, St. Louis, MO) or Sybr Green I (Trevigen, Gaithersburg, MD) staining. The slides were examined and images were captured using Nikon fluorescent microscope and the Leica digital DM 4000b fluorescent microscope equipped with image analysis software. Approximately 60 comet images were taken for each condition. DNA damage was measured by the tail length [53] using Comet Score software available from http://www.scorecomets.com/

6-4PP and CPD dimers

Removal of pyrimidine (6-4) pyrimidone photoproducts (6-4 photoproducts) and cyclobutane pyrimidine dimers (CPDs) were measured by immunoslot blot assay [54, 55]. For this experiment HaCaT keratinocytes were grown in DMEM +5% FBS, in 60 mm dishes, in duplicates, until they have reached 70% confluency. Cells were treated with melatonin or its metabolites at the concentration of 5×10^{-5} M, or ethanol (vehicle, dilution 5:1,000) for 30 before the UVB exposure (50 mJ/cm^2) and for 3 h or 6 h after the exposure. Cells were harvested and DNA was isolated (DNeasy, Qiagen), heat-denatured at 100°C for 10 minutes, applied to a Hybond nitrocellulose membrane (Amersham) using a vacuum-driven slot blot apparatus (100 ng and 50 ng/slot for (6-4) photoproduct and CPD analysis, respectively), and fixed by baking for 1 h at 80°C. Membranes were incubated with mouse monoclonal antibodies specific for 6–4 photoproducts (1/10,000) and CPDs (1/10,000) (Cosmo Bio Co. Cat# NMDND002 and Cat#NMDNDOO1, respectively). Peroxidase-conjugated anti-mouse secondary antibody was used at a dilution of 1/10,000 in blocking buffer. Equal loading of DNA was confirmed by DAPI (Invitrogen) staining.

CPDs in cultured normal keratinocytes were visualised using immunofluorescence. Briefly, HEKn were plated onto chamber slides, treated with melatonin or its metabolites at the concentration of 5×10^{-5} M, or ethanol (vehicle, dilution 5:1,000) for 24 h prior UVB exposure, and further for 3 h after the exposure. Following the manufacturer protocol, cells were stained with anti-CPD (clone TDM2) (Cosmo Bio Co Ltd., Tokyo, Japan) (dil 1:100) and subsequently with goat anti-mouse IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA) (1:100). Nuclei were stained red with Vectashield mounting media with propidium iodide (Vector Laboratories, Burlingame, CA). Stained cells were imaged with a fluorescence microscope. Pictures recorded were analysed using ImageJ software (NIH free download).

Immunofluorescence for p53

Keratinocytes were plated onto chamber slides in duplicates. Cells were treated with melatonin, or its metabolites at the concentration of 5×10^{-5} M, or ethanol (vehicle, dilution 5:1,000) for 24 h prior UVB exposure and for 3 h for detection of p53 or phosphor-p53 Ser-15 or 16 h for detection of phosphor p53 Ser-46 [43]. Immunofluorescence and analysis are performed as described previously [56]. Cells were further fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature and washed three times with 0.1% Triton X-100 (BioRad, Hercules, CA) in PBS to permebealize membrane. Blocking was performed in 10% FBS in PBS for 1 h at RT after quenching of endogenous peroxidase with 1% H_2O_2 (BioRad, Hercules, CA) in PBS. Cells were incubated in primary antibodies diluted in blocker 1:100 overnight at 4°C as follows: anti-p53 (ab4060, Cell Signaling, Danvers, MA), phospho p53 Ser-15 (9284 Cell Signaling, Danvers, MA) or anti-p53 (phosphor S-46) (ab131348, Abcam, Cambridge, MA). The following day cells were washed and incubated with secondary antibody Alexa-Fluor 488 goat anti-rabbit IgG (Invitrogen Molecular Probes, Eugene, Oregon, USA) diluted in blocker 1:100 for 1 h at RT. After washing with PBS, nuclei were stained red with Vectashield mounting media with propidium iodide (Vector Laboratories, Burlingame, CA). Stained cells were imaged with a

fluorescence microscope. Pictures recorded were analysed using ImageJ software (NIH free download).

Reactive oxygen species (ROS) assays

ROS levels were measured using a fluorescence method [57]. Keratinocytes were plated onto 96-well plate and treated with melatonin, or its metabolites at the concentration of 5×10^{-5} M, or ethanol (vehicle, dilution 5:1,000) for 24 h. After preincubation with CM-H2DCFDA dye, cells were irradiated with 200 mJ/cm^2 of UVB. After 2 h of incubation cells were washed with PBS. The generation of ROS was determined by measuring the fluorescence at 480 nm excitation and 528 nm emission, on SpectraMax M2e instrument (Molecular Devices, Sunnyvale, CA). Data are presented as percentile of control (EtOH-treated cells).

Nitric oxide (NO$^-$) assays

Nitrite levels (NO$^-$) levels were measured using Griess reagent (1% sulfanilamide-0.1% N-1-naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid) (Sigma, St. Louis, MO) as previously described [57]. Keratinocytes were treated with melatonin Cells were treated with melatonin, or its metabolites, at the concentration of 5×10^{-5} M, or ethanol (vehicle, dilution 5:1,000) for 24 h. After incubation, cells in 1xPBS were irradiated with the UV of different intensity: 0, 25, 50, and 75 mJ/cm^2. After incubation cells were harvested by scraping. Cells pellets were further centrifuged at 3000 g for 5 min, supernatant was collected and mixed with an equal amount of Griess reagent. The generation of NO was determined by measuring the absorbance at 540 nm in a spectrophotometer of purple azo compound formed from the reaction between nitrates formed in samples and Griess reagent. Potassium nitrate (Sigma, St. Louis, MO) dilutions, ranging from 0–35 μM, were used to create standard curve. Data are presented as concentration of nitite (μmol) per protein amount (mg), and further as percentile of control (EtOH-treated cells).

H$_2$O$_2$ assays

Hydrogen peroxide (H$_2$O$_2$) levels were measured using a luminescence method [58]. Keratinocytes were treated with melatonin, its derivatives at the concentration of 5×10^{-5} M, or ethanol (vehicle, dilution 5:1,000) for 24 h. After incubation, cells in 1xPBS were irradiated with the UVB of different intensity: 0, 25, 50, and 75 mJ/cm^2. The generation of H$_2$O$_2$ was determined by measuring the luminescence of luminol (Sigma, St. Louis, MO) by H$_2$O$_2$ that is released by keratinocytes 1–2 min until 1 h after UV exposure. Aliquots of PCS from each dish were mixed with lumiol and horse radish peroxidase (Sigma, St. Louis, MO) in respiratory buffer and luminescence was measured in a Turner Luminometer (TD20/20) (Promega, Madison, WI). The specificity of the reaction was determined by treating separate UV-irradiated cells with 300 units/mL of catalase (Sigma, St. Louis, MO), which degraded H$_2$O$_2$ to H$_2$O and O$_2$. Data are presented as concentration of H$_2$O$_2$ (pmol) per 0.1 million cells and further as percentile of control (EtOH treated cells).

Reduced glutathione (GSH) assay

Reduced glutathione (GSH) levels were measured using a fluorometric method [57]. Keratinocytes were treated with melatonin, or its metabolites at the concentration of 5×10^{-5} M, or ethanol (vehicle, dilution 5:1,000) for 24 h. After incubation, cells were irradiated with the UVB of different intensity: 25, 50, or 75 mJ/cm^2. After UV exposure cells were further incubated for 1 h. Cells were harvested with trypsinization, washed with 1x PBS-EDTA, pH8. After centrifugation, cell pellets were resuspended in ice cold 5% meta-phosphoric acid (MPA) (Sigma, St. Louis, MO), sonicated, and centrifuged at 12,000 rpm for 5 min. After collection of supernatant, aliquot were taken for protein determination. Supernatant was further mixed with 1x PBS-EDTA buffer and OPAME (o-phthaldialdehyde (Sigma, St. Louis, MO) in methanol (Fisher, Pittsburgh, PA) and borate buffer (potassium-tetraborate, Sigma, St Lois, MO). Mixtures are further aliquoted in 96-well plate, incubated for 15 min at RT and reduced GSH levels were determined by measuring the fluorescent signal at 350nm excitation and 420nm emission, on SpectraMax M2e instrument (Molecular Devices, Sunnyvale, CA). Data are presented as percentile of control (EtOH-treated cells).

Statistical analysis

Data are presented as means ± SEM and were analyzed with Student's t-test (*) and appropriate post hoc test (for more than two groups), using Microsoft Excel and Prism 4.00 (GraphPad Software, San Diego, CA). Statistically significant differences are denoted in the figures and corresponding figure legends.

Results

UVB-induced damage was investigated in human epidermal keratinocytes cultured in vitro. HEKn cells were preincubated with melatonin and its derivatives at graded concentrations from 10^{-13} to 10^{-5} M prior to and after UVB irradiation at doses of 25, 50, or 75 mJ/cm^2. It had been shown previously that treatment with melatonin prior UV exposure protects cells more from UVB [41, 59]. MTS was used as biological test of cellular viability. There was a significant reduction in cell viability and cell number after UVB exposure (data not shown), which was significantly reduced by treatment with melatonin or its derivatives. Figure 1 shows the strong dose-dependent protective effect of melatonin and its derivatives with maximal protection achieved at ligand concentration of 10^{-5}M. NAS and melatonin exhibited lower potency than AFMK, 6-OHM or 5-MT. The data were presented after subtraction from non-irradiated cells, since melatonin and its derivatives have anti-proliferative effect on normal human keratinocytes; thus, fully recovered cells have 0 on y-axis

UVB irradiated human keratinocytes produce ROS and induce DNA damage [1], which can be prevented by melatonin, as tested in HaCaT cells [40, 41]. To determine if melatonin or its metabolites also prevent cell damage and have a protective role, UVB-irradiated keratinocytes, both HEKn and HaCaT, were treated with or without melatonin or its metabolites, were tested for oxidative stress markers i.e., ROS including NO and H_2O_2 formation, GSH levels, CPD dimers formation, and DNA damage.

Figure 2 shows significant reduction (30–50%) in ROS levels produced by UVB irradiated keratinocytes compared with control in cells treated with melatonin or its derivatives. All metabolites had a similar potency in ROS reduction compared to melatonin in both keratinocytes, HEKn and HaCaT (Fig 2 A and B).

Melatonin decreases the toxicity of NO [60]. To test the ability of melatonin metabolites to reduce the amount of NO, HaCaT keratinocytes were incubated with melatonin or its metabolites for 24 h before the UVB exposure. Cells in PBS were irradiated with UVB doses of 25, 50, or 75 mJ/cm^2. Cells were harvested by scraping and collected after 1–2, 30, 45, and 60 min of irradiation to measure azo compounds formed in the reaction between NO and Griess reagent. Release of NO from irradiated cells is compared to non-irradiated cells. There was no NO release in non-irradiated cells in the presence or absence of melatonin or its metabolites (Fig 3). Keratinocytes produced NO in response to UVB irradiation [1] in a UVB-dose dependent manner (Fig 3, insert). The production of NO occurred shortly after irradiation, reaching a peak after 30 min, and returning to low levels after 1 h (data not shown). Treatment of cells with melatonin or its metabolite before UVB irradiation reduced NO production. The most prominent decrease in NO occurred 30 min after UVB exposure (Fig 3). The strongest inhibition of NO release was observed at 50 mJ/cm^2 (Fig 3). Melatonin and AFMK strongly inhibited NO after 25 or 50 mJ/cm^2 (p<0.001). Other metabolites showed strong inhibition of NO after 25 mJ/cm^2, 5-MT and NAS (p<0.001) and 6-OHM (p<0.05), but were much weaker after 50 mJ/cm^2, 5-MT and NAS (p<0.05). Only melatonin inhibited NO after 75 mJ/cm^2 of UVB (p<0.05) (Fig 3).

Melatonin scavenges H$_2$O$_2$ directly [26]. To test the ability of melatonin metabolites to scavenge H$_2$O$_2$ produced in cells, HaCaT keratinocytes were incubated with melatonin and its metabolites for 24 h before the UVB exposure. Cells in PBS were irradiated with UVB doses of 25, 50, or 75 mJ/cm^2. Supernatants were collected after 1–2, 30, 45, and 60 min of irradiation to measure the release of H$_2$O$_2$. Release of H$_2$O$_2$ from irradiated cells was compared to non-irradiated cells. While there was no H$_2$O$_2$ release in non-irradiated cells (Fig 4), keratinocytes produced H$_2$O$_2$ in response to UVB irradiation [1], and this production is UVB dose-dependent (Fig 4, insert). The production of H$_2$O$_2$ occurred 1–2 min after irradiation, reaching a peak after 30 min, and returned to low levels after 1 h (data not shown). Treatment of cells with melatonin or its metabolite reduced H$_2$O$_2$ release induced by UVB. This decrease was the most prominent 30 min after UVB exposure, when H$_2$O$_2$ release reached its peak (Fig 4). The strongest inhibition of H$_2$O$_2$ release was observed at the lowest UVB dose (25 mJ/cm^2), while the lowest inhibition was at the highest UVB dose (75 mJ/cm^2) (Fig 4). AFMK exhibited the strongest inhibitory effect on H$_2$O$_2$ release, followed by melatonin and 6-OHM, while the weakest effect was seen after treatment with 5-MT or NAS. The inhibition of H$_2$O$_2$ release was observed at 25 and 50, but not after 75 mJ/cm^2 (Fig 4). AFMK showed the strongest inhibition of H$_2$O$_2$ by approximately 70% (p<0.001). Melatonin and 6-OHM exhibited stronger inhibition of NO after 25 mJ/cm^2, (p<0.001), when compared to 50 mJ/cm^2 inhibition, ~30–50% (p<0.05). Interestingly, 5-MT and NAS showed stronger activity after higher dose of UVB, ~20% (p<0.01 at 50 mJ and p<0.05 at 25 mJ/cm^2). None of the compounds tested inhibited H$_2$O$_2$ production at a UVB intensity of 75 mJ/cm^2 (Fig 4).

Exposure to UVB causes immediate reduction of GSH [61]. Previously it was documented that melatonin can increase reduced GSH in human vascular endothelial cells [62] The effects of melatonin and its metabolites on the relative GSH content after treatment of keratinocytes for 24 h before and 1 h after UVB exposure are shown in figure 5. The level of reduced GSH decreased with the exposure to UVB light of different intensities (Fig 5, insert). At different intensities of UVB light tested (25, 50, or 75 mJ/cm^2), melatonin and its metabolites caused a significant rise in reduced GSH after 1 h of UVB exposure. GSH content increased by 25% after 25 mJ/cm^2, and 30–70% after 50, or 75 mJ/cm^2 exposure (fig 5). All melatonin metabolites exhibited statistically significant increase in the levels of GSH ($p<0.05$-$p<0.001$).

To measure DNA repair capacity, elimination of DNA damage in the form of UVB-induced 6-4 photoproducts was measured by immunoslot-blot analysis, as previously described [54]. Nucleotide excision repair (NER) is the principal mechanism for repair of UVR generated (6-4) photoproducts; the majority of 6-4PP was removed by 6 h after irradiation (Fig 6A). Densitometric analysis of the bands (Fig 6B) showed that melatonin or melatonin derivatives enhanced the rate of removal of 6-4PP dimers in HaCaT cells. This effect was similar at both concentrations tested (10^{-5} M vs 10^{-6} M) (data not shown). NAS had no effect on removal of 6-4PP dimers from keratinocytes irradiated with UVB (data not shown).

Immunoslot-blot analysis failed to show any effect on CPDs after 3 or 6 h (not shown). Therefore, reduction in CPDs following UVB exposure in keratinocytes was assessed by immunohistochemistry followed by image analysis (Fig 7). UVB-irradiated HEKn cells were immediately treated with or without melatonin or its derivatives for 3 h, and CPD-positive cells were evaluated with CPD-specific antibody. CPD-positive cells were not detected in untreated cells (Fig 7A). CPD-staining intensity of UVB-irradiated cells was weaker in cells treated with melatonin or its derivatives (Fig 7B,C), as compared to untreated cells, indicating that melatonin or its derivatives might accelerate the repair of UVB-induced CPD in keratinocytes. The differences between melatonin, melatonin metabolite-treated cells, and non-treated cells after the exposure to UVB was statistically significant and more pronounced after UVB of 25 mJ/cm^2 ($p<0.01$ or $p<0.001$) (Fig 7B), then after 50 mJ/cm^2 ($p<0.05$) (Fig 7C). Melatonin, AFMK, and 6-OHM were the strongest inhibitors of CPD formation, but they showed no significant effects in cells exposed to UVB dose of 75 mJ/cm^2 (data not shown).

To quantify the extent of the DNA damage, we employed the Comet assay which allows DNA of the damaged cells to migrate from the nucleus in agarose under an electric current, and further using fluorescent microscopy for visualisation of DNA migration. We observed an extensive DNA damage when HEKn cells were exposed to UVB (Fig 8, insert). There was a significant difference between UVB-induced DNA damage in control cells (non-treated) vs. cells treated with melatonin or NAS ($p<0.01$) or 6-OHM and 5-MT ($p<0.05$), but not AFMK, as measured by mean comet tail moment (Fig 8).

Tumor suppressor p53 has very important role in DNA repair of UV-induced DNA damage [63]. Following UV irradiation or oxidative stress p53 accumulates in nucleus (Fig 9A).

While phosphorylation of p53 can occur at different sites, the most important are the Ser-15 site, promoting accumulation and activation of p53 and DNA repair, and Ser-46, regulating apoptosis following DNA damage [64]. Previously it had been shown that melatonin induces a p53-dependent DNA damage response phosphorylation at Ser-15 [45, 46]. Therefore, we tested the expression of p53 and its phosphorylated forms (Ser-15 and Ser-46) in HEKn keratinocytes irradiated with UVB and treated with melatonin and its metabolites. UVB-irradiated cells showed positive nuclear signal (Fig 9A). P53 accumulation was proportional to the UVB intensity (Fig 9B).

To determine whether a reduction of UVB-induced cell damage by treatment with melatonin and its metabolites allows keratinocytes with DNA damage to survive, we compared p53 accumulation in irradiated-treated vs. non-treated cells. The phosphorylation of keratinocytes of the Ser-15 site occurs earlier (3 h after UVB exposure), while phosphorylation of the Ser-46 site occurs later (12 h after UVB exposure) (data not shown), which is in accordance with previous report in MCF7 breast carcinoma cells [43]. While melatonin and its metabolites did not change significantly the expression of p53 and p53 phosphorylated form (Ser-46) (data not shown), the expression of p53 phosphorylated form of the Ser-15 increased after treatment with melatonin or its metabolites (Fig 10). The strongest response was after AFMK treatment ($p<0.001$), followed by melatonin, 6-OHM and NAS ($p<0.01$ or $p<0.05$), and 5-MT ($p<0.05$) after exposure to 25 mJ/cm^2 UVB (Fig 10A) or 50 mJ/cm^2 (figure 10B). At UVB 75 mJ/cm^2, only 6-OHM and 5-MT showed effect on p53 levels ($p<0.05$) (Fig 10C).

Discussion

UVB damages skin keratinocytes causing: oxidative DNA damage and the production of reactive oxygen species, including hydrogen peroxide and nitric oxide, and changing levels of glutathione [1]. UVB also plays a role in melatonin production in the skin [12], which further protects skin from oxidative damage induced by UV irradiation [12, 18, 30, 40, 65, 66]. Melatonin's protective actions from UVR-induced oxidative damage is a results of its stimulation of antioxidative enzyme activities and scavenging free radicals [12, 18, 30, 40, 65, 66]. Stress promotes production of melatonin [11] and it as well as its metabolites scavenge free radicals and stimulate antioxidative enzymes, including glutathione peroxidase, glutathione reductase, and superoxide dismutase [5, 8, 26, 35, 67–73]. Under physiological conditions melatonin can stimulate the production of radical oxygen species (ROS) in a small amount, while when the cell is exposed to stress, melatonin protects cells by limiting the leakage of ROS out of cell [32] and maintaining normal membrane potential [42, 74], thus preventing cell injury. To determine if melatonin metabolites also prevent cell damage and exhibit protective role, UVB-irradiated keratinocytes treated with or without melatonin or its metabolites were tested for oxidative stress markers, including NO and H_2O_2 formation, GSH levels, CPD dimers formation, and DNA damage. Herein, we report protective effects of melatonin and its metabolites in to reducing oxidative cell damage in human keratinocytes elicited by UVB, as determined by attenuation of each of these oxidative stress parameters (ROS, NO, H_2O_2, rGSH, CPD). Thus, like melatonin, its derivatives also attenuate oxidative damage induced by UVB.

Nitric monoxide (NO⁻) toxicity is created by its coupling of respiratory produced O2, thus forming a non-radical, but highly toxic peroxynitrite anion (ONOO⁻), which may be further metabolized to –OH [6]. Melatonin also detoxifies NO [60], thereby lowering ONOO⁻ formation. Since melatonin promotes the activities of antioxidative enzymes: including glutathione peroxidase (GPx) and glutathione reductase (GPr) [71, 75], damage by free radicals is limited when H_2O_2 is metabolised to non-radical species and glutathione is maintained in its reduced form. GSH is a potent antioxidant and plays an important role in preventing protein damage [61]. In general, melatonin plays an important role in maintaining glutathione homeostasis [76]. The current studies are thus in agreement with other reports showing that melatonin increases concentration of intracellular GSH [62]. Moreover, we show that melatonin metabolites are equally as potent as melatonin in increasing GSH levels (Figure 5), decreasing ROS levels (Fig 2), NO (Fig 3), and H_2O_2 levels (Fig 4) in UVB-exposed keratinocytes.

Irradiation of human keratinocytes with UVB resulted in the generation of H_2O_2 (Fig 4). H_2O_2, although a weakly toxic ROS, can be converted to highly toxic -OH [6]. The levels of released H_2O_2 were markedly reduced by melatonin or its metabolites, suggesting that they modulate the UV-induced oxidative stress in keratinocytes. Numerous studies have shown that melatonin prevents DNA damage by scavenging H_2O_2 directly or indirectly through production of AFMK [26]. The scavenging of H_2O_2 happens rapidly, within first few minutes and is melatonin mediated; while a slower phase of scavenging is mediated by AFMK and other metabolites [26]. Our data show that melatonin and its metabolites limit H_2O_2 generationin UVB-irradiated keratinocytes in a UVB dose-dependent manner, suggesting that the best scavenging effects occur after exposure to a lower dose of UVB (Fig 4). This data is in accordance with the findings that melatonin's action is proportional to the amount of stress the cell is under [67, 77]. The strongest scavenging effects are seen 30 min after UVB exposure, when the release of H_2O_2 by keratinocytes was the highest (Fig 4).

In this study, the comet assay was used to examine the actions of melatonin and its metabolites on DNA repair (Fig 8). Comet assay involves single-cell electrophoresis that enables the DNA of the damaged cells to migrate from the nucleus, thus forming a tail moment. It is clear that melatonin or its derivatives reduced UVB-induced DNA-damage (Fig 8). Our data also shows that UV-induced DNA repair in keratinocytes was also enhanced by melatonin and its derivatives (Fig 6 and 7).

Previously, it was shown that melatonin prevents UVB-mediated keratinocyte damage [41]. Tumor suppressor protein p53 mediates the cell response to stress by activating cell-cycle arrest or apoptosis [63]. Different stressors, including gamma and UV irradiation, hypoxia, some chemicals, induce DNA damage and activation of p53 [78]. The extend of DNA damage and the rise in p53 are proportional [78]. UV irradiation causes transient DNA damage in the cell, such as DNA breaks and excision repair of the DNA dimers, thus inducing a moderate elevation in p53 level and cell-cycle arrest that allows repair to occur [63]. Phosphorylation plays an important role in p53 functioning, including binding to DNA [79, 80]. Phosphorylation of p53 occurs at different sites. P53 phosphorylation at the Ser-15 and Ser-20 sites promotes accumulation and activation of p53 and DNA repair, while p53 phosphorylation at the Ser-46 site closely regulates apoptosis after DNA damage [64].

Melatonin promotes phosphorylation of p53 at Ser-15 position, thus activating p53 protein and consecutively inhibiting cell growth, preventing accumulation of DNA damage and promoting antitumor activity [14, 43]. This action of melatonin is mediated by its MT1 and MT2 receptors [45]. Since phosphorylation of p53 at Ser-46 site is required for p53-induced apoptosis, we tested the ability of melatonin and its metabolites to promote p53-phosphorylation, thus inducing p53-dependent apoptosis. Upregulation of nuclear p53 and phosphorylated form of p53 after UVB was measured by immunohistochemistry and image analysis. As indicated by the preliminary data, there was no difference in p53 expression in sham-irradiated keratinocytes treated with vehicle compared with cells treated with melatonin or its derivatives, as measured by immunohistochemistry (data not shown). Exposure to UVR significantly augmented p53 expression in human keratinocytes compared with sham vehicle (control cells) (Fig 9). This effect was further enhanced in UVB-exposed keratinocytes treated with melatonin and its derivatives as shown in figure 10. Melatonin and its derivatives significantly induced the level of phosphorylated form of p53 at the Serine 15 site (Fig 10). Thus, our data indicate that the protective effects of melatonin and its metabolites against UVB induced damage are associated with p53-phosphorilation at Ser-15.

Although the role melatonin and AFMK play in protection against oxidative damage from different stressors including UVB, is well established [35, 81–84], we believe our findings are novel in the areas of skin biology and photobiology. Thus, using immortalized and most importantly normal epidermal primary keratinocytes we compared protective role of both melatonin and its metabolites including NAS, 6-OHM, AFMK, and 5-MTover wide range of parameters (oxidative stress, cell viability, DNA damage, p53 phosphorylation) that was differential and dependent on the molecule structure in the context of parameters tested. Taking into consideration that melatonin is both produced and metabolised in epidermal keratinocytes [13, 31], we propose that activity of the local melatoninergic system define protection of human epidermis against UVB exposure by attenuating DNA and metabolic damage via several mechanism, including reduction in H_2O_2 levels, reduction of NO levels, promotion of GSH, enhancement of DNA repair, and promotion of keratinocytes survival, in a context dependent fashion.

Acknowledgments

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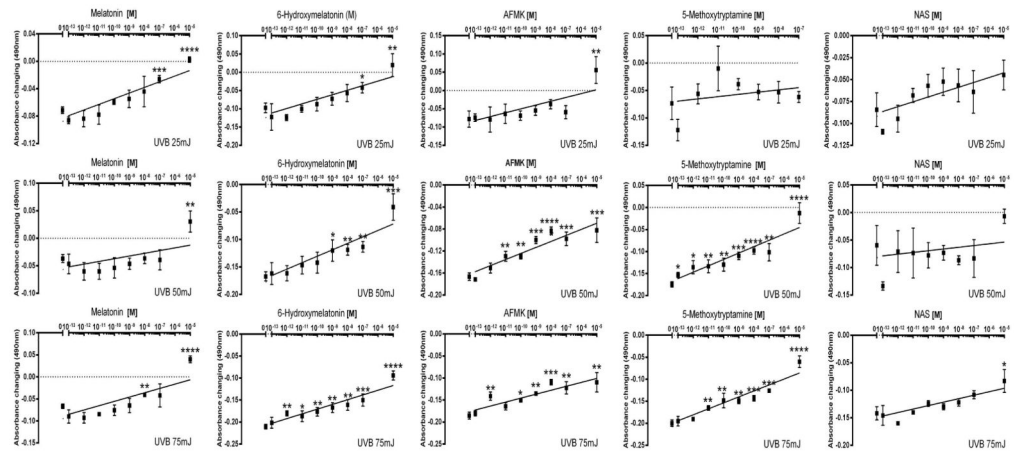
Figure 1.
Melatonin, NAS, 6-OHM, AFMK, and 5-MT increase viability of HEKn keratinocytes exposed to UVB. Data are calculated by subtracting viability from non-irradiated cells which means we considered the anti-proliferative effect of melatonin and its derivatives on this cell. Data were analyzed using student t-test, * p<0.05, ** p<0.01, and *** p<0.001.
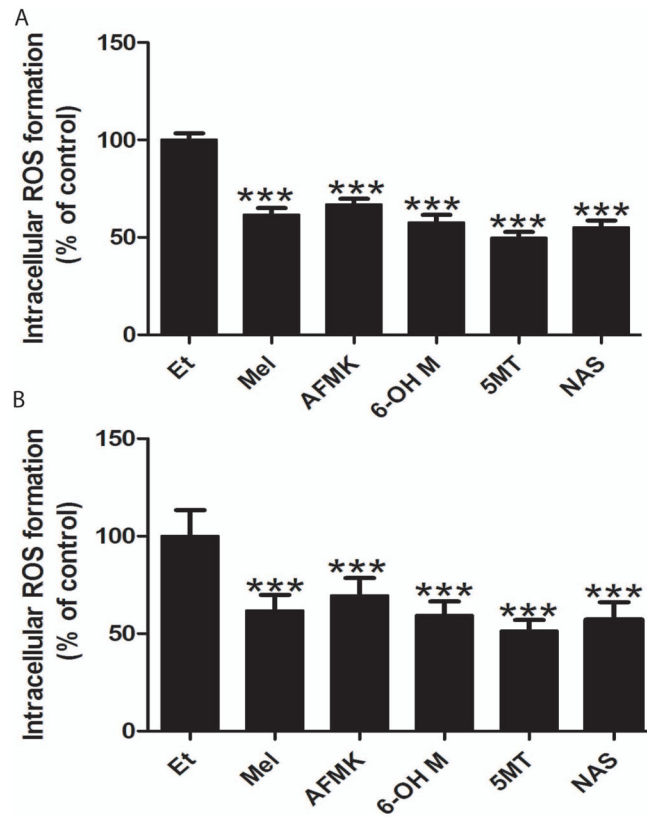
Figure 2.
Melatonin, NAS, 6-OHM, AFMK, and 5-MT decrease UVB-induced ROS generation. UVB-irradiated (200 mJ/m^2) keratinocytes (HEKn (A) and HaCaT (B)) were treated with melatonin or its metabolites for 24 h prior UV irradiation and further for 2 h post UV at the concentration 5×10^{-5} M. Data were analyzed using student t-test, *** p<0.001.
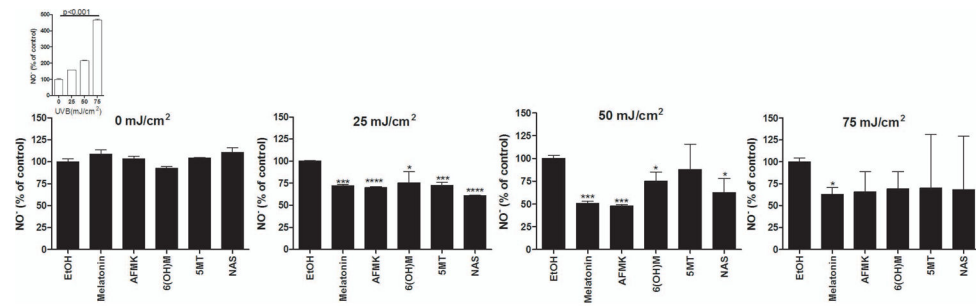
Figure 3.
Melatonin, NAS, 6-OHM, AFMK, and 5-MT reduce levels of NO-produced by UVB-irradiated keratinocytes. HaCaT keratinocytes were treated with melatonin, AFMK, 6-OHT, 5-MT, NAS, or EtOH for 24 h and subsequently irradiated with 0, 25, 50, or 75 mJ/cm^2 of UVB. NO-produced in keratinocytes was determined 30 min after UVB irradiation. The differences in NO-production under different UVB intensities are shown as an insert. Data are presented as percent and were analyzed using t-test, * p<0.05 and *** p<0.001.
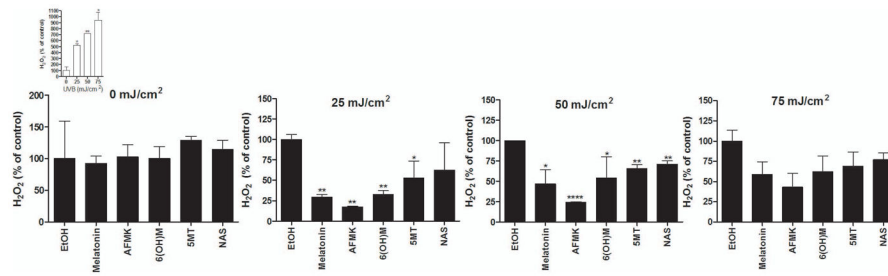
Figure 4.
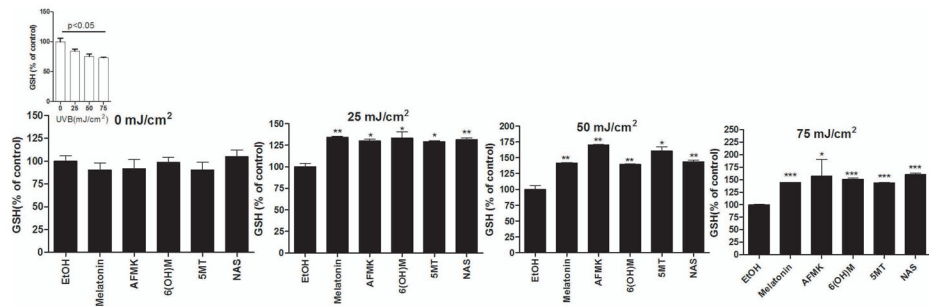Melatonin, NAS, 6-OHM, AFMK, and 5-MT reduce levels of H_2O_2 produced by UVB-irradiated HaCaT keratinocytes. Keratinocytes were treated with melatonin, AFMK, 6-OHT, 5-MT, NAS, or EtOH for 24 h and further irradiated with 0, 25, 50, or 75 mJ/cm^2 UVB. H_2O_2 produced in keratinocytes was determined 30 min after UVB irradiation. The differences in H_2O_2 production under different UVB intensities are shown as an insert. Data are presented as percent of control and were analyzed using t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.
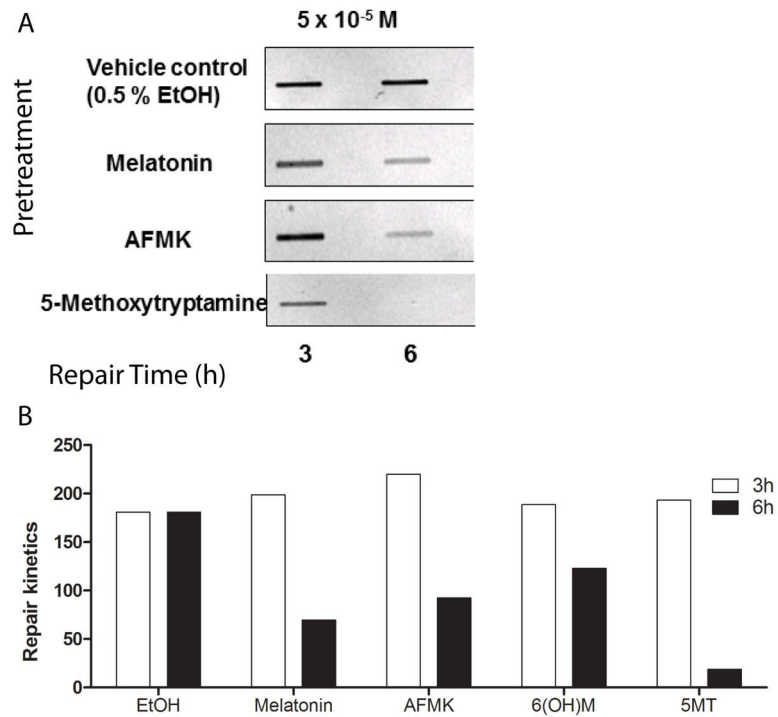
Figure 5.
Melatonin, NAS, 6-OHM, AFMK, and 5-MT increase GSH in UVB-exposed keratinocytes. HaCaT keratinocytes were treated with melatonin, AFMK, 6-OHM, 5-MT, NAS, or EtOH for 24 h and further irradiated with 0, 25, 50, or 75 mJ/cm^2 UVB. GSH produced in keratinocytes was determined 1 h after UVB irradiation. The differences in GSH production under different UVB intensities are shown as an insert. Data are presented as percentile of control and were analyzed using t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 6.
Melatonin, NAS, 6-OHM, AFMK, and 5-MT treated keratinocytes exhibit enhanced UVR-induced DNA repair. HaCaT cells were treated with melatonin or its metabolites for 30 min before UVB exposure (50 mJ/cm^2) and for 3 or 6 h after. DNA was measured by immunoslot blot analysis following UVB exposure. The relative DNA repair efficiency was compared to non-treated cells (EtOH).
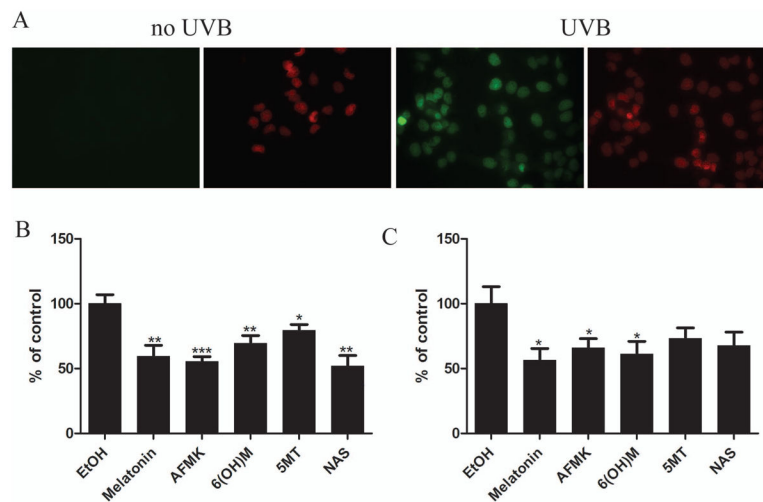
Figure 7.
Melatonin, NAS, 6-OHM, AFMK, and 5-MT treated keratinocytes decrease CPD formation after UVB exposure. HEKn keratinocytes were treated with melatonin or its derivative for 24 h before UVB exposure. Cells were exposed to UVB intensities of 25 or 50 mJ/cm^2 and immediately treated again with melatonin or its derivatives for 3 h. Cells were further fixed and stained with anti-CPD antibody (green) and propidium iodine (PI) (red) (A). Stained cells were imaged with a fluorescence microscope, fluorescence intensity was analysed using ImageJ software, and data are analysed using Graph Pad Prizm after 25 mJ/cm^2 (B) and 50 mJ/cm^2 (C). Data are presented as percentile of control and were analyzed using t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
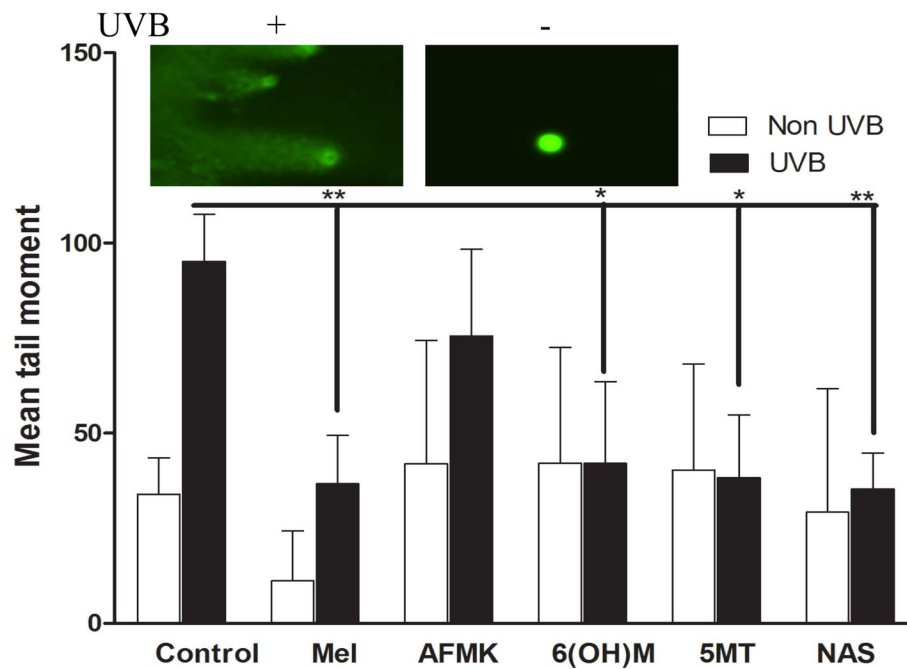
Figure 8.
Melatonin, NAS, 6-OHM, AFMK, and 5-MT enhance repair of DNA damage induced by UVB as indicated by comet assay. HEKn keratinocytes were treated with melatonin or its derivatives before and after UV-irradiation (200 mJ/cm^2) for 3 h. Photographs show comets in UV-irradiated and non-irradiated keratinocytes. UV caused strand breaks in DNA which migrated in the electrophoretic field to form comet tails. Tail moment is used to measure DNA damage. Data are analysed using student t-test and labelled as * $p<0.05$, ** $p<0.01$.
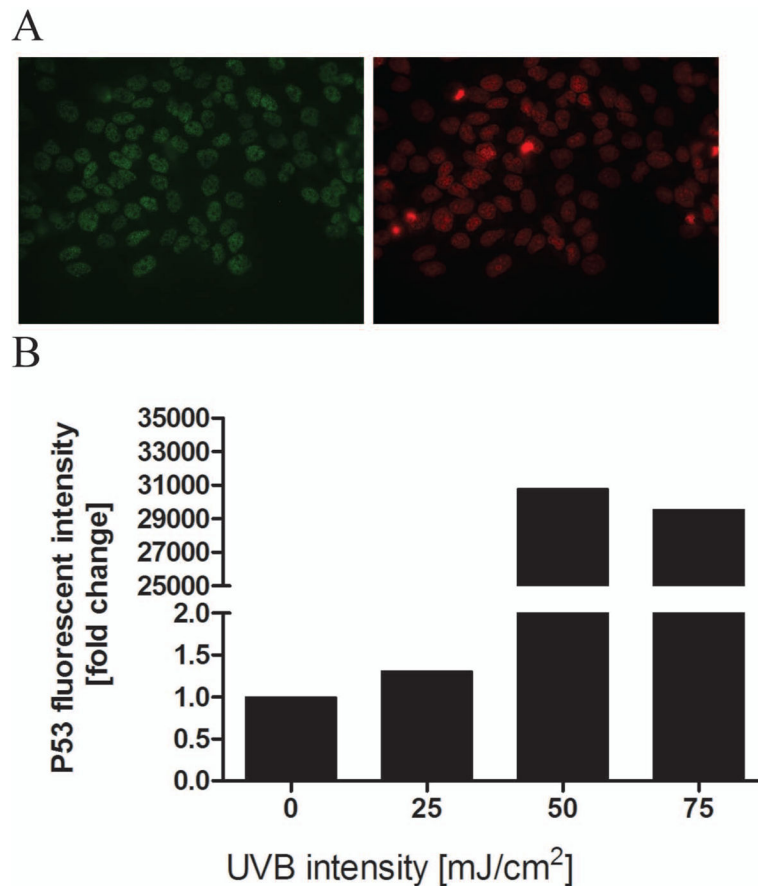
Figure 9.
UVB irradiation increases p53 levels. HEKn keratinocytes were exposed to UVB intensities of 0, 25, 50, or 75 mJ/cm^2. Cells were subsequently fixed and stained with anti-p53 antibody (green) and propidium iodine (PI) (red) (A). UVR-irradiated cells showed positive nuclear signal for p53 (A). Stained cells were imaged with a fluorescence microscope; fluorescence intensity was analysed using ImageJ software. Data are analysed using Graph Pad Prizm and expressed as fold change compared to control (0 mJ/cm^2). P53 levels increased proportionally to UVB intensity (B). Data are presented as percentile of control and were analyzed using t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
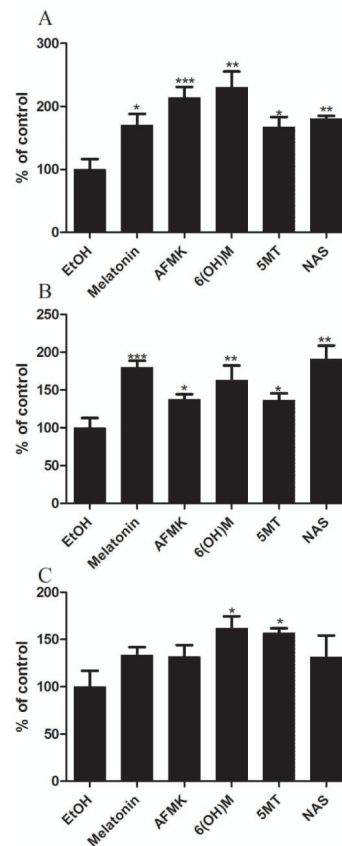
Figure 10.
Melatonin, NAS, 6-OHM, AFMK, and 5-MT treated HEKn keratinocytes show increase of p53 phosphorylated at Serine 15 after UVB exposure. Keratinocytes were treated with melatonin or its derivative for 24 h before UVB exposure. Cells were exposed to UVB intensities of 25 (A), 50 (B), or 75 mJ/cm^2 (C) and immediately treated again with melatonin or its derivatives for 12 h. Cells were further fixed and stained with anti-phosphorylated p53S15 antibody. Stained cells were imaged with a fluorescence microscope; fluorescence intensity was analysed using ImageJ software. Data are analysed using Graph Pad Prizm. Data are presented as percentile of control and were analyzed using t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$