1 *Article*

2 Anti-Wrinkle Mechanism of Melatonin in UVB
3 Treated HaCaT Keratinocytes and Hairless Mouse via
4 Inhibition of ROS and Sonic Hedgehog Mediated
5 Inflammatory Proteins

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11

12 **Abstract:** Though melatonin is known to improve ultraviolet B (UVB)-induced oxidative damage
13 and inflammatory conditions via blockade of nuclear factor (NF)-κB, interleukin (IL)-6, there is no
14 report on anti-wrinkle effect of melatonin to date. Hence in the present study, anti-wrinkle
15 mechanism of melatonin was elucidated in UVB treated HaCaT keratinocytes and hairless mice.
16 Herein melatonin protected against a radical initiator tert-Butyl hydroperoxide (t-BOOH) induced
17 reactive oxygen species (ROS) production, matrix metalloprotease 1 (MMP-1) and cytotoxicity in
18 HaCaT keratinocytes. Also, melatonin suppressed the expression of sonic hedgehog (SHH) and GLI
19 for hedgehog signaling, p-NF-κB, cyclooxygenase (COX-2), p-ERK for inflammatory responses in
20 UVB treated HaCaT keratinocytes. Furthermore, melatonin protected skin from wrinkle formation,
21 transdermal water loss in hairless mice irradiated by UVB for 8 weeks. Notably, melatonin
22 prevented against epidermal thickness and dermal collagen degradation in UVB irradiated hairless
23 mice by Hematoxylin & Eosin and Masson's trichrome staining**.** Taken together, these findings
24 suggest that melatonin reduces wrinkle formation via inhibition of ROS/SHH and inflammatory
25 proteins such as NF-κB/COX-2/ERK/MMP1.

26 **Keywords:** Melatonin, UVB, HaCaT keratinocytes, collagen, ROS, Hedgehog, Cox-2, MMP-1
27

28 1. Introduction

29   Facial wrinkles are known one of the most prominent features for skin aging [1]. Skin aging is
30 usually classified into intrinsic (chronological) and extrinsic (photoaging) aging. Extrinsic aging
31 shows photoaged skin with roughly wrinkles mainly due to the exposure to sunlight including
32 ultraviolet B, while intrinsic aging is induced by the aging genetic factors with the pale and smoothly
33 wrinkle formation [2]. It is well documented that the loss of skin elasticity and degradation of elastic
34 fibers and collagen are usually shown in aged or wrinkled skin [3, 4]. Previous evidences reveal that
35 reactive oxygen species (ROS) in dermal fibroblast and epidermal keratinocyte produce matrix
36 metalloproteinases (MMPs) to induce collagen degradation leading to skin aging, since MMPs
37 degrade the extracellular matrix, including collagen fibers, and thus contributes to wrinkle
38 formation[5]. In particular, UVB exposure upregulates the expression of MMP-1, -3, and -9 in human
39 epidermis to induce wrinkle formation [6].
40   Thus, recently many phytochemicals such as magnolo l [7], abietic acid [8], Galla Chnensis
41 extract [9] and green tea pholyphenols [10] were reported to block skin damage due to chronic UVB
42 irradiation. Also, melatonin was reported to protect human keratinocytes from UVB-induced
43 oxidative stress and DNA damage. Nevertheless, the anti-wrinkle mechanism of melatonin still

44 remains unclear to date. Thus, in the present study, anti-wrinkle molecular mechanism of melatonin
45 was investigated in t-BOOH or UVB treated human keratinocytes and UVB irradiated SKH-1 hairless
46 mice.
47

48 **2. Results**

49 *2.1. Melatonin protected against t-BOOH induced ROS production and cytotoxicity in HaCaT keratinocytes*

50   It was well known that tert-Butyl hydroperoxide (t-BOOH) has been used as a radical
51 polymerization initiator to induce cell damage[11]. To find out protective effect of melatonin (Fig. 1A)
52 in t-BOOH induced cytotoxicity in HaCaT keratinocytes, MTT assay was conducted. As shown in
53 Figure 1b, c, t-BOOH and UVB (30 mJ/cm^2) exerted cytotoxicity better than melatonin in HaCaT
54 keratinocytes. However, melatonin significantly suppressed t-BOOH induced cytotoxicity (Figure 2a)
55 and ROS production at the concentrations of 1 and 2 mM in HaCaT keratinocytes (Figure 2b).
56

57   **(a)**   **(b)**   **(c)**
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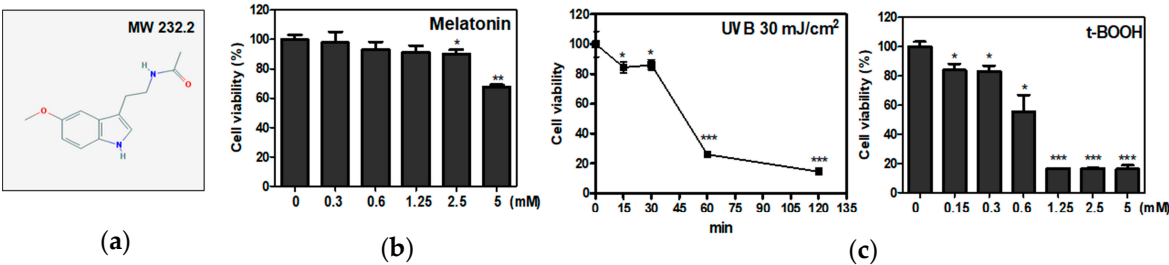
59 **Figure 1.** Effect of Melatonin, t-BOOH and UVB on the cytotoxicity in HaCaT cells. (a) Chemical
60   structure of melatonin. Molecular weight = 232.2 c (b, c) HaCaT cells were seeded onto 96-well
61   microplates at a density of 1X10^4 cells/well and treated with various concentrations of Melatonin (0,
62   0.3, 0.6, 1.25, 2.5 or 5 mM) or t-BOOH (0, 0.15, 0.3, 0.6, 1.25, 2.5 or 5 mM) for 24 h. HaCaT cells were
63   exposed to UVB (30 mJ/cm2) for 15 min, 30 min, 60 min or 120 min. Data represent means ± SD.
64   *p<0.05, ** P<0.01 and *** P<0.001 versus untreated control.

65   **(a)**   **(b)**
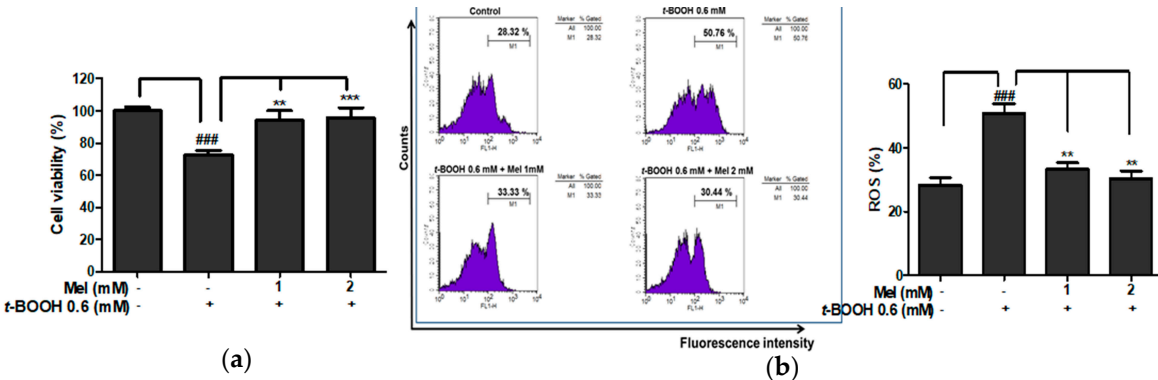
66 **Figure 2.** Melatonin suppressed t-BOOH induced cytotoxicity and ROS production in HaCaT
67   keratinocytes. (a) Cells were pretreated with melatonin for 24h and then with t-BOOH (0.6 mM) for
68   6h. (b) ROS generation (%) was measured using ROS-sensitive fluorometric probe 2,7-
69   dichlorofluorescein diacetate (DCFDA) by flow cytometric analysis. Data represent mean ± SD. **
70   p<0.01 versus treated t-BOOH, ### p<0.001 versus untreated Control.

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74   *2.2. Melatonin attenuated the mRNA expression of MMP-1 in t-BOOH or UVB induced HaCaT*
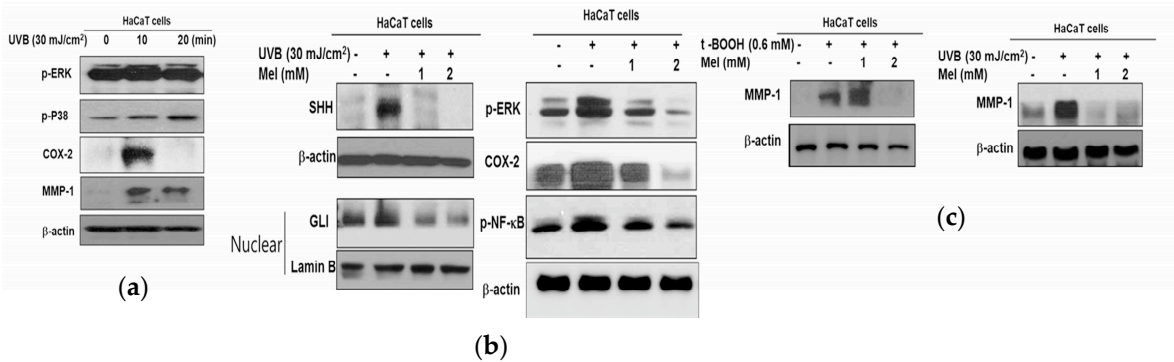75   *keratinocytes*

76   There are accumulating evidences that the expression of MMPs was upregulated in t-BOOH
77   induced HaCaT keratinocytes [8, 12]. Also, UVB-induced photoageing is known to be mediated by
78   increased expression of MMP-1 and collagen degradation [13, 14]. To determine the effects of t-BOOH
79   and UVB on MMP-1 expression, qRT-PCR assay was performed. As shown in Figure 3a, melatonin
80   reduced the mRNA expression of MMP-1 in t-BOOH treated HaCaT keratinocytes. Also, melatonin
81   suppressed the mRNA expression of MMP-1 and increased the expression of pro-collagen in UVB
82   treated HaCaT keratinocytes (Figure 3b).
83

(a)   (b)
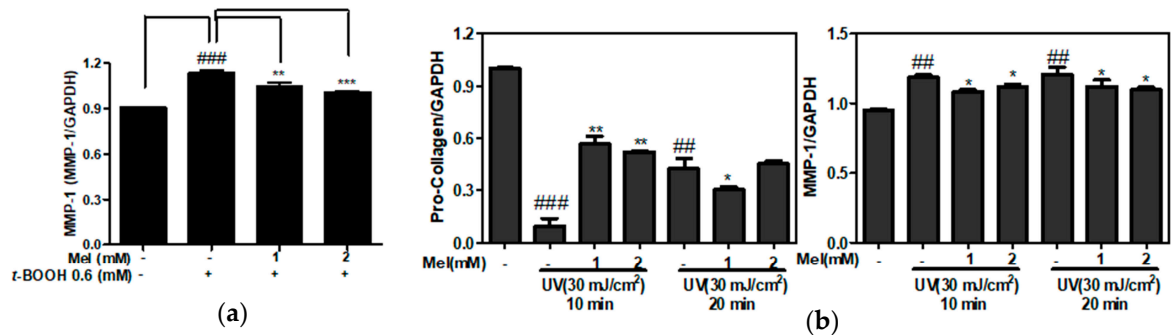
86   **Figure 3.** Melatonin attenuated the mRNA expression of MMP-1 and pro-collagen in t-BOOH and
87   UVB induced HaCaT keratinocytes. (a) Cells were pretreated with melatonin for 24h and then with
88   t-BOOH (0.6 mM) for 6h. Data represent mean ± SD. *** $p<0.001$ and ** $p<0.01$ versus treated t-
89   BOOH, ### $p<0.001$ versus untreated Control. (b) Cells were pretreated with melatonin for 24h and
90   then irradiated with UVB (30 mJ/cm2) for 10 min or 20 min. Data represent mean ± SD. *** $p<0.001$,
91   ** $p<0.01$ and * $p<0.05$ versus irradiated UVB, ### $p<0.001$ and ## $p<0.01$ versus untreated Control.

92   *2.3. Melatonin effectively suppressed the UVB-induced hedgehog signaling and MMP-1 related proteins in*
93   *HaCaT keratinocytes*

94   To determine whether melatonin inhibits UVB-induced MMP-1(photoaging marker) related
95   proteins, Western blotting was performed. After the exposure to UVB 30 mJ/cm^2 for 10 min or 20 min,
96   MMP-1 related proteins (p-ERK, Pp-38, COX-2 and MMP-1) were significantly affected at UVB 30
97   mJ/cm^2 for 10 min (Figure 4a). To determine whether the intracellular signal mechanism of melatonin,
98   we examined the effects of melatonin on the hedgehog signaling pathway, since hedgehog signaling
99   [15] (SHH/GLI) plays a key role in inducing MMP-1. Consistently, melatonin blocked UVB-induced
100   SHH and GLI expression at 1 and 2 mM (Figure 4b). Also, Figure. 4B showed that the phosphorylation
101   of NF-κB, ERK and COX-2 was increased by UVB irradiation. Nonetheless, melatonin reduced p-NF-
102   κB, p-ERK and COX-2 in the UVB-treated HaCaT keratinocytes. Also, melatonin suppressed the
103   expression of MMP-1 in t-BOOH or UVB treated HaCaT keratinocytes (Figure 4c).
104

(a)   (b)   (c)

105

106

107 **Figure 4.** Melatonin attenuated the expression of SHH, GLI and MMP-1 related proteins in t-BOOH and
108 UVB induced HaCaT keratinocytes. (a) HaCaT cells were exposed to UVB (30 mJ/cm2) for 10 min or 20 min
109 and subjected to Western blotting for p-ERK, PP38, COX-2, MMP-1 and β-actin. (b, c) Cells were pretreated
110 with melatonin for 24h and then irradiated with UVB (30 mJ/cm2) for 10 min and subjected to Western
111 blotting for SHH, GLI (nuclear fraction), p-ERK, COX-2, p-NF-κB, MMP-1, β-actin and Lamin B. Cells were
112 pretreated with melatonin for 24h and then with t-BOOH (0.6 mM) for 6h.

113 *2.4. Melatonin prevented against loss of water on the dorsal skin of SHK1 hairless mice exposed to UVB*

114 It was well known that UVB can induce skin damages such as melanogenesis, erythema,
115 epidermal thickness in epidermis, leading to wrinkle formation in dermis[16]. As shown in Figure 5a,
116 melatonin significantly blocked the loss of water on the dorsal skin of SHK-1 hairless mice exposed
117 to UVB at 8 weeks following UVB treatments, while the loss of water was accentuated in UVB alone
118 treated control compared to untreated normal control.
119

120 *2.5. Melatonin suppressed the degree of wrinkles and epidermal thickness induced by UVB irradiation on the*
121 *dorsal skin of SHK1 hairless mice*

122 The degrees of wrinkles were observed on the dorsal skin of UVB irradiated SHK-1 hairless mice
123 by SILFLO casting method, while those were reduced in melatonin treated SHK-1 hairless mice under
124 UVB exposure as shown in Figure 5b. Consistently, H&E staining showed epidermal thickness
125 induced by UVB was blocked by melatonin treatment and Masson's trichrome staining revealed that
126 melatonin treatment maintained blue color for collagen staining compared to UVB alone treated
127 control in the dermis of the skin of UVB irradiated SHK-1 hairless mice as shown in Figure 5c,d.
128

(a)   (b)   (c)   (d)
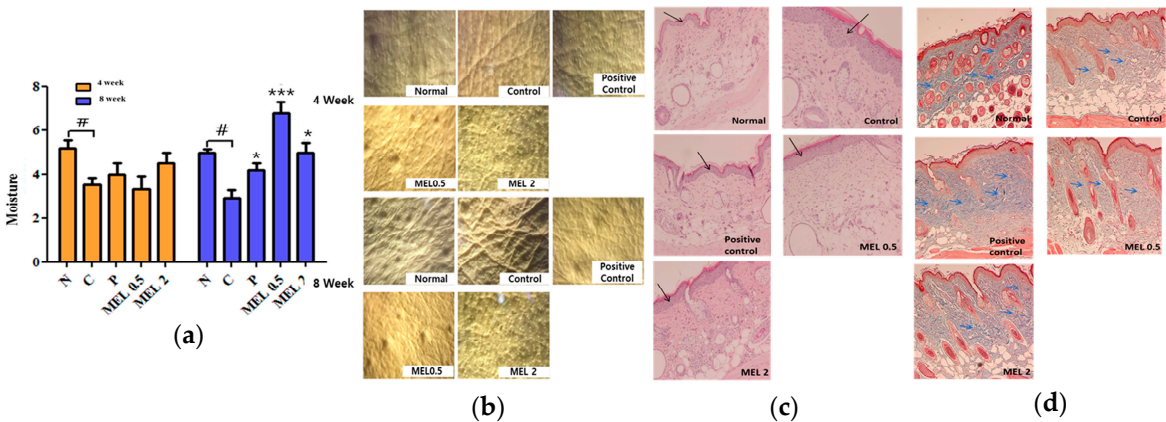
131 **Figure 5.** Melatonin significantly suppresses water loss, wrinkles and collagen fibers in the dorsal skin
132 of SKH 1 hairless mice. (a) Effect of melatonin on water loss in the dorsal skin of SKH 1 hairless mice.
133 N, Normal; C, Control +UVB irradiated; P, Positive control: Retinoic acid 2 mg/kg + UVB; MEL 0.5,
134 Melatonin 0.5 mg/kg +UVB; MEL 2, Melatonin 2 mg/kg +UVB. Data represent mean ± SD. *** p<0.001
135 and * p<0.05 versus irradiated UVB, #p<0.05versus untreated Control. (b) Effect of melatonin on
136 wrinkles in the dorsal skin of SKH 1 hairless mice. Melatonin suppressed the degree of induced by
137 UVB irradiation on the dorsal skin of SHK1 hairless mice. Images of skin replicas were casted on the
138 dorsal skin surface of mouse by using SILFLO. (c) Hairless mouse skin was stained with hematoxylin
139 and eosin (x100). Epidermis thickness was measured under light microscopy. Black arrow
140 (epidermis). (d) Collagen fiber formation was evaluated using Masson's trichrome staining. Collagen
141 fiber bundles are shown in blue area (Blue arrow) (x100).

3. Discussion

Melatonin (N-acetyl-5-methoxytryptamine) is known to have multi-biological activities to regulate circadian rhythms, and work as a hormone or neurotransmitter, antiinflammatory agent, and antioxidant. Especially, melatonin suppressed ROS production in rat lens[17] and IL-3 stimulated leukocytes[18] and also enhanced the survival of UVB treated keratinocytes by suppressing apoptosis[19].

Herein, anti-wrinkle mechanism of melatonin was elucidated in t-BOOH or UVB treated HaCaT keratinocytes and UVB treated hairless mice.

Melatonin suppressed ROS production and cytotoxicity in free radical initiator t-BOOH treated HaCaT keratinocytes, implying melatonin protects HaCaT keratinocytes from t-BOOH induced cytotoxicity, since ROS play a pivotal role in skin damage by UVB-irradiation.

UVB-induced photoageing is initiated by production of ROS [20, 21], which activate several receptors for IL-1, keratinocyte growth factor and tumor necrosis factor (TNF)-α [22, 23]. Activation of these receptors induces downstream signaling pathways of mitogen-activated protein kinases (MAPKs) such as p38, JNK and ERK, which leads to an increase of the nuclear transcription such as NF-κB and AP-1[22, 24, 25]. Additionally, activation of transcription factor NF-κB induces the expression of inflammatory cytokines such as COX-2, IL-6 and MMPs [26, 27]. Continuous UVB exposure induces expression of MMPs, which degrades collagen by collagen breakdown as well as inhibition of procollagen synthesis [28, 29]. Interestingly, melatonin suppressed the expression of COX-2, p-ERK, p-NF-κB and MMP-1 and increased the expression of procollagen in UVB induced HaCaT cells, indicating melatonin inhibits inflammatory cytokines and photoaging markers, leading to anti-wrinkle effect in skin.

Accumulating evidences reveal that Hh signalling pathway regulates normal growth but its abnormal activation can promote cancer progression and epidemal development [30-33]. Therefore, to identify the molecular mechanism associated with UVB-induced photoageing, the role of Hedgehog (Hh) signaling was evaluated in UVB treated HaCaT cells. Here, melatonin reduced the expression of UVB-induced SHH as a Hh ligand and GLI as a transcriptional effector of Hh pathway, demonstrating melatonin exerts anti-photoaging effect via inhibited Hh signaling pathway.

It was well documented that UVB induces skin damages leading to wrinkle formation due to collagen degradation in the dermis [34, 35]. Here, melatonin protected skin from wrinkle formation, transdermal water loss in UVB treated hairless mice comparable to positive control retinoic acid, indicating melatonin prevents against wrinkle formation in the dermis possibly via inhibition of collagen degradation. Consistently, Masson's trichrome staining revealed that melatonin treatment maintained blue color staining for collagen and reduced epidermal thickness compared to UVB alone treated group in the dermis of the skin of UVB irradiated SHK-1 hairless mice, implying protective effect of melatonin on wrinkle formation by collagen degradation.

Collectively, melatonin suppressed ROS production and the expression of MMP-1in t-BOOH-induced HaCaT keratinocytes. Also, melatonin suppressed the expression of UVB-induced MMP-1 and MMP-1 related proteins (inflammatory proteins) and increased pro-collagen in HaCaT keratinocytes and reduced the expression of hedgehog signaling proteins, protected against epidermal thickness, transdermal water loss, collagen degradation and wrinkle formation on the dorsal skin of UVB treated hairless mice. Overall, these findings demonstrate that melatonin protects against UVB induced oxidative skin damage including wrinkle formation via inhibition of ROS, MMP-1 and hedgehog signaling pathway.

4. Materials and Methods

4.1. Chemicals

Melatonin (N-acetyl-5-methoxytryptamine) (M5250) was purchased from Sigma (St. Louis, MO).

193 *4.2. Cell culture*

194   Human skin keratinocyte cell line HaCaT cells (CRL-2404) were purchased from American Type
195 Culture Collection (ATCC). The cells were grown in Dulbecco Modified Eagle Medium (DMEM)
196 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum, antibiotics (penicillin, 100 U/mL;
197 streptomycin, 100 µg/mL) and antimycotic (amphotericin B 0.25 µg/mL).

198 *4.3. UVB irradiation*

199   HaCaT cells (70–80% confluency) were treated with melatonin (0, 1 or 2 mM) for 24 h. After
200 washed with phosphate-buffered saline (PBS; pH 7.4), the cells were irradiated with 30 mJ/cm^2 of
201 UVB (312 nm) for 10 min by CL-1000 Ultraviolet crosslinker.
202
203 *4.4. Cytotoxicity assay*

204   The cytotoxicity of melatonin was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-
205 diphenyltetrazolium bromide (MTT) assay. In brief, HaCaT cells (1×10^4 cells/well) were seeded onto
206 96-well culture plate and exposed to various concentrations of melatonin for 24 h. The cells were
207 incubated with MTT (1mg/mL) (Sigma Chemical) for 2 h and then treated with MTT lysis solution
208 overnight. Optical density (OD) was measured using a microplate reader (Molecular Devices Co.,
209 USA) at 570 nm. Cell viability was calculated as a percentage of viable cells in melatonin treated
210 group versus untreated control.

211 *4.5. RT-qPCR analysis*

212   RT-qPCR was performed with the LightCycler TM instrument (Roche Applied Sciences,
213 Indianapolis, IN) with following primers, human MMP-1 forward: 5'-
214 CATGACTTTCCTGGAATTGG -3'; reverse- 5'-CCTGCAGTTGAACCAGCTAT-3' (Bioneer, Daejeon,
215 Korea), human pro-collagen forward: 5'-CAGGCAAACCTGGTGAACA -3' ; reverse -5'-
216 CTCGCCAGGGAAACCTCT -3' human GAPDH-forward5'-GCACCGTCAAGGCTCTAGAAC-
217 3';reverse-5'-GGATCTCGCTCCTGGAAGAT -3' (Bioneer, Daejeon, Korea).

218 *4.6. Western blotting*

219   HaCaT Cells were pretreated with melatonin for 24h and then with t-BOOH (0.6 mM) and UVB
220 (30 mJ/cm2), lyzed in lysis solution (50mM Tris–HCl, pH 7.4, 150mM NaCl, 1% Triton X-100, 0.1%
221 SDS, 1mM EDTA, 1mM Na$_3$VO$_4$, 1mM NaF, and 1× protease inhibitor cocktail) on ice, and spun down
222 at 14,000×*g* for 20min at 4°C. The supernatants were collected and quantified for protein
223 concentration by using RC DC protein assay kit (Bio-Rad, Hercules, CA, USA), The proteins samples
224 were separated on 4–12% NuPAGE Bis–Tris gels (Novex, Carlsbad, CA, USA) and transferred to a
225 Hybond ECL transfer membrane for detection with antibodies for p-ERK, Pp-38, COX-2, MMP-1,
226 SHH and GLI (Cell signaling Technology, Beverly, MA, USA) Lamin B (Santa Cruz Biotechnologies,
227 Santa Cruz, CA, USA), and β-actin (Sigma, St. Louis, MO, USA).

228 *4.7. Nuclear fraction for GLI*

229   HaCaT cells were pretreated with melatonin for 24h and then exposed to UVB irradiation (30
230 mJ/cm2). Then nuclear fraction of the cells was prepared using NE-PER nuclear and cytoplasmic
231 extraction reagents (Thermo, Rockford, IL, USA) based on the manufacturer's protocol for Western
232 blotting with GLI.

233 *4.8. Measurement of reactive oxygen species (ROS) generation*

234   2,7-Dichlorofluorescein diacetate (DCFH-DA) was used to measure the levels of ROS production.
235 HaCaT cells were pretreated with melatonin for 24h and then with t-BOOH (0.6 mM) for 6h. Then 5
236 µM DCFH-DA for 30 min at 37°C. Fluorescence intensity was measured by FACSCalibur (Becton
237 Dickinson, Franklin Lakes, NJ, USA).

238   *4.9. Animals and treatments*

239   Fifty female SKH-1 mice (6 week old) were purchased from Samtako Biokorea (Samtako Biokorea,
240   Osan, Kyunggido, Republic of Korea) and housed at IK Science cage under consistent condition
241   (temperature: 22 ± 1℃, humidity: 55 ± 3%, 12 h light/ dark cycles). All experiments were performed
242   in accordance with the guidelines established by Kyung Hee University. Mice were acclimated for
243   one week and randomly divided into 5 groups (n = 10) during eight week experimental period;
244   normal group (N); control group (only UVB treated group; three times a week by UV-1000 (Dongseo
245   Science, Korea)); positive control retinoic acid (Sigma Aldrich, USA) group (2 mg/kg) (B); melatonin
246   0.5 mg/kg (MEL 0.5); melatonin 1 mg/kg (MEL 1). Melatonin was orally administered to the mice of
247   each group daily for 8 weeks, while saline was administered to Normal and control group and
248   retinoic acid was i.p injected in positive control group three times a week for 8 weeks.
249   UVB irradiation schedule is as follows:
250   1) 0 week: 60 mJ/cm^2 for 200sec, three times a week (1 minimal erythema dose; M.E.D)
251   2) 1week: 120 mJ/cm^2 for 400sec, three times a week (2 M.E.D)
252   3) 2~3weeks: 180 mJ/cm^2 for 600sec 1week: 120 mJ/cm^2 for 400sec, three times a week (3 M.E.D)
253   4) 4~5 weeks: 240 mJ/cm^2 for 800sec, three times a week (4 M.E.D)
254

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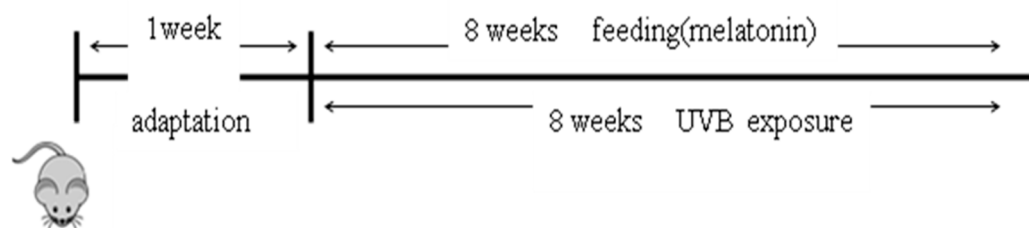
256   *4.10. Observation of skin wrinkle formation*

257   The condition of skin wrinkle formation on the dorsal skin of hairless mice exposed to UVB was
258   observed at 4 weeks and 8 weeks, the replicas were casted on the dorsal skin surface of mouse by
259   using SILFLO (Flexico developments LTD. Tokyo, Japan) and then photographed by camera(Nikon
260   D90, Japan).

261   *4.11. Measurement of skin moisture*

262   After mice were kept in the room under the condition of 21.5±2 ℃ and relative humidity 40±5%
263   for 30 min. Skin moisture was measured in melatonin treated mice by using Corneometer CM 820
264   (Courage - Khazake, Koln, Germany).
265

266   *4.12. Histological analysis*

267   Dorsal skin was obtained from melatonin or retinoic acid treated groups and control group mice
268   by biopsy, fixed in 4% paraformaldehyde, dehydrated in ethanol and then embedded in paraffin.
269   Approximately 10μm-thick sections were deparaffinized and stained with hematoxylin-eosin (H&E)
270   and Masson's trichrome staining. Stained slides were then photographed using a light microscope
271   (ZEISS Observer D2, Germany).

272   *4.13. Statistical analysis*

273   For statistical analysis of the data, Sigmaplot version 12 software (Systat Software Inc., San jose,
274   CA, USA) was used. All data were expressed as means ± standard deviation (SD). Student t-test was
275   used for comparison of two groups. The statistically significant differences were set at p values of
276   <0.05 between control and melatonin treated groups.
277

Author Contributions: Park EK and Lee HJ designed and performed several experiments. Lee H, Kim JH, Hwang JS supported experiments. Kim SH supervised this experiments and wrote MS.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

UVB	Ultraviolet B
t-BOOH	*tert*-Butyl hydroperoxide
ROS	Reactive Oxygen Species
MMP-1	Matrix metalloprotease 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

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