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Blue light impairs the repair of UVB-induced pyrimidine dimers in a human skin model

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1 **ABSTRACT**

2 In the recent years, interest is growing in the biological cutaneous effects high-energy visible
3 light (400-450 nm). In the present study, we explored the impact of blue light (BL) on the repair
4 of pyrimidine dimers, the major class of premutagenic DNA damage induced by exposure to
5 sunlight. We unambiguously demonstrate that the exposure of *in vitro* reconstructed human
6 epidermis to environmentally relevant doses of BL strongly decreases the rate of repair of
7 cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts induced by a
8 subsequent UVB irradiation. Using the highly sensitive and specific liquid chromatography /
9 tandem mass spectrometry assay, we did not observe induction of pyrimidine dimers by BL
10 alone. Finally, we showed that application, during the BL exposure step, of a formula containing
11 a new filter, named TriAsorB and affording BL photoprotection, prevented the decrease in DNA
12 repair efficiency. These results emphasize the potential deleterious effects of BL on DNA repair
13 and the interest in providing adequate skin protection against this wavelengths range of sunlight.

14

15 INTRODUCTION

16 Ultraviolet (UV) irradiation leads to harmful effects including skin aging and skin cancer due to
17 increased production of cellular reactive oxygen species and by direct DNA damage. Damaged
18 DNA, if not properly repaired, is a source of mutations, and interferes with many cellular
19 mechanisms such as replication, transcription, and cell cycle. Recent studies have highlighted
20 the impact of the visible light on the skin. An action spectrum of visible light in HaCat
21 keratinocytes showed that the deleterious effect decreased in the order violet > blue > green >
22 red (1). These observations demonstrate the major role played by high-energy visible light
23 spectral range (400-450 nm). Large amounts of information have been in particular gathered on
24 blue light (BL) and revealed damaging effects on cutaneous cells mainly by inducing an
25 oxidative stress. In keratinocytes cultured *in vitro*, violet and BL have been shown to induce
26 oxidative stress, mitochondrial dysfunctions and blockade of autophagy and DNA damage (2,
27 1, 3). Similar observations were made in melanocytes (4). Another interesting feature of visible
28 light is that it is more abundant and penetrates deeper into the skin layers than UV. BL have
29 been implicated in erythema, hyperpigmentation and premature skin aging (5-9). Induction of
30 oxidative stress is also documented (2). This evidence that BL is active in skin raises the concern
31 about a possible worsening impact of BL on effect of UV.

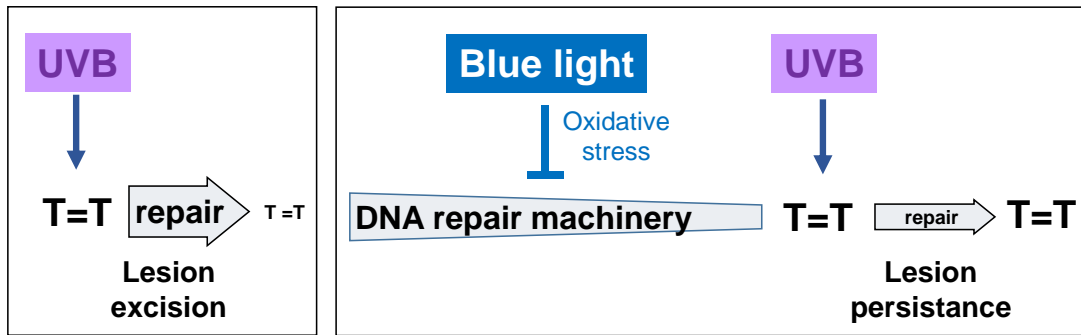
32 A major issue in the deleterious effect of sunlight is its genotoxicity, which is directly linked to
33 induction of skin cancer. *In vitro*, BL has been shown to damage DNA by oxidation processes
34 (10, 11, 1, 3). The latter are triggered by BL mediated photosensitization reactions involving
35 endogenous chromophores (2, 12). Superoxide anion is the main species produced (2), likely
36 through excitation of flavins (13). BL has also been shown to induce chromosome aberrations

37 *in vitro* (3). BL-induced DNA damage is yet approximately 3 orders of magnitude less efficient
38 at damaging DNA than UVB at inducing cyclobutane pyrimidine dimers (CPDs) (10, 11). More
39 recently, a study on human volunteers showed that the induction of dark CPDs was efficient at
40 380 nm but not in the visible light range (14). These results strongly suggest that the DNA
41 damage induced by BL do not significantly contribute directly to the initiation of skin cancer.
42 However, BL may be indirectly genotoxic by preventing the repair of DNA damage induced by
43 other wavelengths ranges, especially carcinogenic UVB.

44 In order to test this hypothesis, we designed a realistic protocol where reconstructed human
45 epidermis (RHE) were first exposed to BL and then to UVB. We further took advantage of the
46 photoprotective efficacy against BL of the new sunfilter Phenylene Bis-Diphenyltriazine
47 (TriAsorB[®]) to unambiguously establish the role of BL. This organic sunscreen has a critical
48 wavelength ≈ 390 nm. The filter effectively absorbs visible light especially in the region from
49 400 to 450 nm bandwidth. The absorbance decreases with increasing wavelength and dropped
50 at about 0.1 at 450 nm with a $\lambda_{Vis-CW} = 594$ nm (15). Moreover, TriAsorB is also able to reflect
51 BL with a peak of reflectance at about 450 nm and a progressive decrease up to 700 nm (15). Its
52 filtering efficacy against BL radiations has been demonstrated by inhibition of the formation of
53 8-oxo-7,8-dihydroguanine in reconstructed human skin after exposure to 80 J.cm^{-2} of BL at
54 427 ± 30 nm. Moreover, TriAsorB formulation as a SPF50⁺ sunscreen in combination with other
55 UV filters prevented cellular photodamage and BL-induced oxidatively generated DNA lesions
56 (9).

57

58 **GRAPHICAL ABSTRACT**



59

60 Exposure of reconstructed human epidermis with blue light decreases the rate of repair of
61 pyrimidine dimers induced in DNA by a subsequent UVB irradiation. This deleterious effect is
62 prevented by application of a sunscreen exhibiting blue light photoprotection.

63

64

66 **MATERIALS AND METHODS**

67 **TriAsorB and SPF50⁺ sunscreen formulations**

68 The commercially available SPF50⁺ sunscreen containing TriAsorB[®] (Laboratoires Eau
69 Thermale Avène, France) is a combination with three other UV filters: diethylamino
70 hydroxybenzoyl hexyl benzoate (Uvinul A+[®], BASF SE), ethylhexyl triazone (Uvinul T150[®],
71 BASF SE) and bis-ethylhexyloxyphenol methoxyphenyl triazine (Tinosorb S[®], BASF SE), as
72 recently reported (9). This sunscreen contains Avène thermal spring water, C12-15 alkyl
73 benzoate, caprylic/capric triglyceride, dicaprylyl carbonate, diethylamino hydroxybenzoyl
74 hexyl benzoate (Uvinul A+), glycerine, ethylhexyl triazone (Uvinul T150), phenylene bis-
75 diphenyltriazine (Triasorb), bis-ethylhexyloxyphenol methoxyphenyl triazine (Tinosorb S),
76 potassium cetyl phosphate, stearyl alcohol, VP/eicosane copolymer, caprylyl glycol, glyceryl
77 stearate, PEG-100 stearate, PPG-1-PEG-9 lauryl glycol ether, glyceryl dibehenate, xanthan gum,
78 benzoic acid, tribehenin, polyacrylate-13, glyceryl behenate, sorbitan isocitrate, polyisobutene,
79 tocopheryl glucoside, polysorbate 20, Red 33, and tocopherol (patented association WO581;
80 WO984).

81 A formulations containing 5% TriAsorB[®] was prepared according the following INCI: Ethylic
82 alcohol, Avène thermal spring water (Avène aqua), C12-15 alkyl benzoate, caprylic/capric
83 triglyceride, dicaprylyl carbonate, glycerin, potassium cetyl phosphate, polyacrylate-13, PEG-
84 100 stearate, stearyl alcohol, VP/eicosane copolymer, phenoxyethanol, glyceryl behenate,
85 glyceryl dibehenate, benzoic acid, xanthan gum.

86 **Growth of reconstructed human epidermis**

87 In this study, the model used was a follicular model of RHE *in vitro*. The epidermis was
88 reconstructed from keratinocytes derived from the outer epithelial sheath of human hair follicles
89 obtained from healthy volunteers who had given informed consent. The 3D reconstruction
90 method was previously described by Guiraud et al. (16) and Bacqueville et al. (17). The outer
91 epithelial sheath cells of human hair follicles are a readily available and non-invasive source of
92 keratinocytes for epidermal reconstruction. Briefly, the cells were isolated from the outer
93 epithelial sheath of the hair follicles and then cultured before seeding on culture inserts
94 (CLS3460, Corning®) immersed in culture medium. The culture inserts are then placed at the
95 air/liquid interface in an incubator at 37°C with 5% CO₂ to induce epidermal differentiation and
96 maturation of the *stratum corneum*. The culture medium is renewed every 24 hours. The RHE is
97 formed in 16 days with a surface area of 1.12 cm².

98 **Blue light and UVB irradiations of skin model**

99 TriAsorB and SPF50⁺ sunscreen formulations were evaluated for BL photoprotection. They
100 were applied at a dose of 2 mg.cm⁻² to the surface of a polymethyl methacrylate (PMMA) plate.
101 This plastic is naturally transparent and has greater light transmission than glass. The PMMA
102 plate is then deposited on the RHE model just before exposure to BL radiation using a blue light
103 LED lamp (Kessil PR160) (15, 9). The power of the LED lamp is 257.4 W.m⁻² and the
104 wavelength is 427±30 nm. The RHE models were exposed to BL at a dose of 60 or 92 J.cm⁻² for
105 a duration of 39 min and 1 hour, respectively.

106 After this BL stress, the PMMA plate was removed and the skin was then exposed to UVB by
107 using a Biosun RMX irradiator (Vilber Lourmat, Germany) equipped with a monochromatic

108 illumination lamp at 312 nm. The lamp power is approximately 3 mW.cm⁻². RHE models were
109 exposed to a single dose of 50 mJ.cm⁻². To assess DNA repair, tissues were finally harvested
110 just after UVB exposure (t0) or 48 hours after the UVB stress (t48h), and all were frozen at -80°C
111 before analysis by liquid chromatography coupled to tandem mass spectrometry (HPLC-
112 MS/MS).

113 All experiments were performed in 3 independent biological replicates (N=3) with n=3 or 4
114 technical replicates for each series (n=3), except for the conditions UVB stress alone (UVB
115 control) and BL stress plus UVB stress (BL + UVB) (N=6, n=3 or 4).

116 **DNA extraction and quantification of photoproducts**

117 DNA was then extracted from the RHE with the DNeasy tissue kit (Qiagen) following the
118 manufacturer instructions. Briefly, samples were grinded in a tissue lyzer with steel beads in
119 buffer ATL. Samples were then incubated for 3 h at 55°C in the presence of proteinase K,
120 followed by a RNA treatment for 2 min. The lysis was completed by a 10 min incubation at
121 70°C with AL buffer. Ethanol was added and DNA trapped on silica gel column. It was washed
122 twice and finally collected in water. The recover fraction was freeze-dried. The residue obtained
123 was enzymatically hydrolyzed and analyzed by HPLC-MS/MS as previously reported (18).

124 **Statistical analyses**

125 Non-parametric analyses were performed on the Prism-GraphPad software (San Diego, CA). A
126 Kruskal-Wallis multiple comparison was first performed. It was followed by comparison
127 between selected conditions by use of a post-hoc Dunn test. Reported results are median ± 25th-
128 75th percentiles range.

129 RESULTS

130 Quantification of photoproducts after UVB and Blue light + UVB exposure

131 Cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts
132 (64PPs) were quantified in the DNA of RHE collected either immediately (t0) or 48 hours after
133 irradiation (t48h) (Table 1). No photoproduct was detected in the DNA of unirradiated RHE or
134 exposed only to BL. The distribution of photoproducts at t0 just after UVB exposure was similar
135 to previous results: i) CPDs are 3 times more frequent than 64PPs, ii) the order of formation of
136 CPDs is $TT > TC > CT$, iii) TC 64PP is more frequent than TT 64PP and iv) very little Dewar
137 valence isomers are produced with UVB (19, 20). The observations at t48h for DNA repair after
138 pure UVB are also those expected, namely that 64PPs are removed from DNA faster than CPDs
139 (21-24). In addition, not all CPDs were repaired with the same efficiency. The decreasing order
140 of removal rate was $CT > TC > TT$ (24). BL exposure at either 60 or 92 J.cm⁻² before UVB
141 irradiation did not affect the level of photoproducts quantified at t0 as inferred from the absence
142 of statistically significant differences. Interestingly, the frequency of pyrimidine dimers was
143 lower 48h post-exposure. These differences were statistically significant for 64PPs under all
144 conditions and for CPDs after pure UVB irradiation. However, statistical significance was not
145 reached for samples exposed to BL before UVB.

146 **Table 1:** Level of photoproducts (dimer per 10^6 normal bases)*. The dose of blue light was either 60 or
 147 92 $J.cm^{-2}$ (BL60 or BL92, respectively). Results were obtained from 6 to 3 biological replicates involving
 148 3 or 4 technical replicates.

dimer	UVB t0	UVB t48h	BL60+UVB t0	BL60+UVB t48h	BL92+UVB t0	BL92+UVB t48h
TT CPD	84.1±31.8	47.1±24.4***	74.1±17.4	67.2±18.8	94.4±39.6	73.3±48.1
TT 64PP	5.7±2.5	0.3±0.3***	6.0±1.5	1.1±1.2**	6.6±2.7	0.9±1.0***
TC CPD	37.9±17.2	22.3±9.0***	39.5±8.6	35.0±11.3	42.8±23.8	38.0±21.1
TC 64PP	35.1±16.8	1.7±2.2***	40.3±18.1	5.5±7.9**	36.2±32.0	3.8±5.3***
CT CPD	12.2±8.9	4.0±4.1***	13.1±11.0	8.8±7.5	10.9±10.7	6.4±6.9

149 * Data are median ± 25th-75th percentiles range. (Statistical significance of the difference between t0
 150 and t48h: ** $p < 0.01$, *** $p < 0.001$)

151

152 Effect of blue light on DNA repair

153 For a better assessment of the impact of BL on DNA repair, repair efficiency was calculated for
 154 each value. Equation (1) was used:

$$155 \quad (1) \quad \%_{\text{repair}} = 100 * (\text{average}(\text{dimer}_{t_0}) - \text{dimer}_{t_{48h}}) / \text{average}(\text{dimer}_{t_0})$$

156 where average(dimer_{t0}) is the average of the level of a photoproduct under specific conditions
 157 at t0, dimer_{t48h} is a value of the level of the same photoproduct and conditions at 48h, and %repair
 158 is the index of repair efficiency.

159 The %_{repair} parameter was calculated for all individual photoproducts (Table 2) as well as for the
 160 sum of either all CPDs or all 64PPs (Fig. 1) following the exposure to pure UVB, BL 60 $J.cm^{-2}$

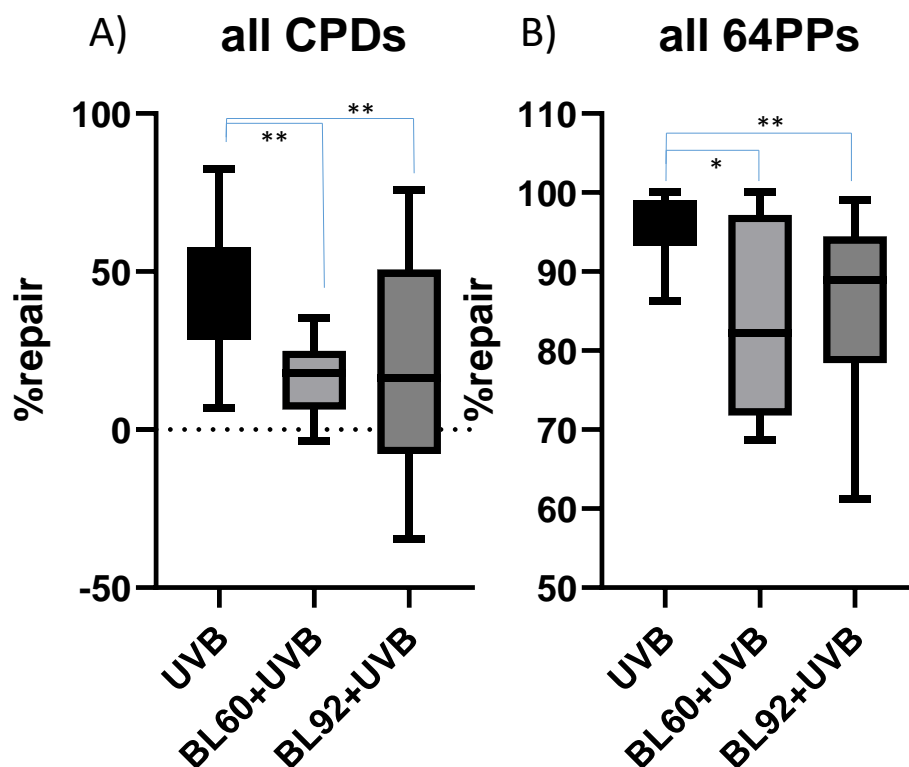
161 + UVB and BL 92 J.cm⁻² + UVB. A statistically significant decrease in the repair efficiency was
 162 observed in all cases, whatever the BL dose applied before UVB irradiation. Remarkably, the
 163 deleterious effects of BL on DNA repair were even observed for 64PPs that are very efficiently
 164 excised. The differences between %_{repair} for the 60 and 92 J/cm² BL treatments did not reach
 165 statistical significance.

166 **Table 2:** Repair efficiency (%_{repair})* for the different exposure protocols. The dose of blue light was either
 167 60 or 92 J.cm⁻² (BL60 or BL92, respectively). Statistical significance of the differences was obtained by
 168 comparison with pure UVB.

dimer	UVB	BL60+UVB	BL92+UVB
TT CPD	42.0±28.0	18.6±29.1*	7.9±71.1*
TT 64PP	94.4±5.6	82.0±19.9*	84.6±16.5***
TC CPD	42.4±23.4	16.4±26.8*	7.7±51.2**
TC 64PP	94.9±6.7	84.0±23.1*	88.8±15.9*
CT CPD	68.6±31.8	36.7±54.6**	49.8±53.4*

169 * Data, expressed in %, are median ± 25th-75th percentiles range. Results were obtained from 6 to 3
 170 biological replicates involving 3 or 4 technical replicates. (Statistical significance of the difference with
 171 respect to pure UVB exposure: * p< 0.05; ** p< 0.01; *** p< 0.001).

172



173

174 **Figure 1.** %_{repair} of A) all CPDs and B) all 64PPs determined in RHE exposed under different conditions.

175 The dose of blue light was either 60 or 92 J.cm⁻² (BL60 or BL92, respectively). Results are median ±

176 25th-75th percentiles range. (Statistical significance of the difference with respect to pure UVB

177 exposure: * p < 0.05; ** p < 0.01).

178

179 Photoprotection against BL-induced DNA repair impairment

180 Quantification of DNA repair was then performed with BL-absorbing/reflecting sunscreens

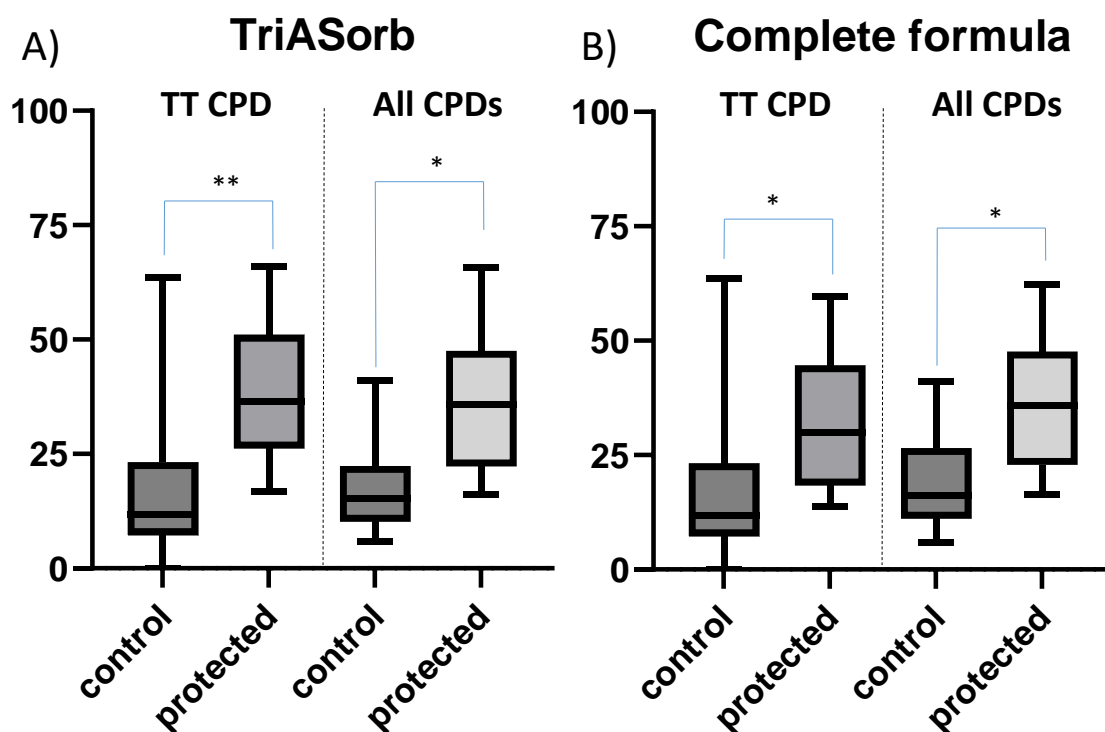
181 spread on a PMMA plate, placed above the RHE only during the BL irradiation. Data were

182 obtained with BL at the 60 J.cm⁻² dose. Both a TriAsorB 5% formulation and a commercial

183 SPF50⁺ sunscreen containing this UVB + UVA + BL filters were investigated for their

184 photoprotective properties against the deleterious effects of BL radiations on DNA repair. A

185 significant improvement of DNA repair was observed for sunscreen formulations. This was well
 186 shown for the DNA repair of TT CPD and the combination of all CPDs (Fig. 2) for which the
 187 efficiency of repair was doubled by using a photoprotection during BL exposure. A protective
 188 effect was also observed for TC CPD with both formulations. Interestingly, a positive effect of
 189 TriAsorB 5% was observed for either TT 64PP or all 64PPs combined.



190
 191 **Figure 2.** Improvement of the repair efficiency (% repair) of TT CPD or all CPDs in the presence of
 192 photoprotectors during the BL exposure. A) unprotected compared to TriAsorb-protected RHE; B)
 193 unprotected compared to complete formula-protected RHE. Reported statistical significance of the
 194 differences was obtained by comparison with sample to BL 60 J.cm⁻² without photoprotection and then
 195 UVB. Data, expressed in %, are median ± 25th-75th percentiles range. Results were obtained from 3
 196 biological replicates involving 3 or 4 technical replicates (statistical significance between
 197 photoprotected and control RHE: * p < 0.05; ** p < 0.01).

198

199 **DISCUSSION**

200 The present results unambiguously demonstrate, for the first time, that BL hampers the repair of
201 pyrimidine dimers in the DNA of reconstituted human skin tissue. This phenomenon was
202 observed on CPDs, the repair rate of which was c.a. 50% in UVB-irradiated sampled and half
203 of this value when a preliminary BL exposure was applied. Interestingly, a statistically
204 significant effect was also obtained for 64PPs for which 95% of the damage was removed after
205 48h from UVB-irradiated RHE. These results provide additional evidence of possible
206 deleterious effects of high energy visible light (400-450 nm) since the LED lamp used in this
207 study was centered at 427 ± 30 nm. In terms of underlying mechanism, the present observations
208 are reminiscent of those made with UVA in a similar experimental design in primary cultures of
209 human skin cells (23, 25). Explanation for this effect of UVA is likely associated with the
210 capacity of these radiations at oxidizing and degrading DNA repair proteins (26-28). In this
211 respect, we can speculate that BL could act as UVA by altering DNA repair process following
212 protein oxidation. Further studies will be required to confirm this hypothesis. It may be added
213 that no pyrimidine dimer could be detected by HPLC-MS/MS in the DNA of RHE exposed only
214 to BL. This result is in agreement with our previous results in human skin (14) but contrasts with
215 that obtained with the less specific comet assay in cultured keratinocytes (3). Other works in cell
216 culture also failed at detecting CPDs at wavelengths higher than UVA (10, 11).

217 The other major result of the present study is that BL exposure of the skin model through a blue
218 light absorbing/reflecting sunscreen specifically prevented decrease in repair capacities. It is
219 now well established that sunscreens do prevent formation of DNA photoproducts, especially at
220 high SFP. This was shown both *in vivo* (29-31), in RHE (9) and *ex-vivo* in skin explants (32-34,

221 17). For example, the sunscreen used in the present work decreased by 95% the yield of
222 pyrimidine dimers in a human skin model exposed to simulated sunlight (9). Yet, a fraction of
223 UV photons is not absorbed/reflected by the photoprotectors. In addition, sunscreens may be
224 washed out and people do not use them permanently even under sunny conditions. Therefore,
225 the formation of DNA damage cannot be completely avoided. Consequently, preventing the
226 decrease of the repair efficiency by sunscreens containing blue light absorbing/reflecting
227 components is an interesting strategy in terms of photoprotection.

228

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330 **TABLES**

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335 **FIGURE CAPTIONS**

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