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Abstract

Numerous epidemiological investigations show that sunlight is carcinogenic to humans and that the use of sunscreen may be effective in decreasing the risk of skin cancer. The biological activity of a sunscreen is evaluated by its ability to protect human skin from erythema as represented by an SPF (Sun Protection Factor). We propose that the sunscreen's protective effect against sunlight-induced genotoxicity, including mutation, should also be taken into account. In this study we examined the protective ability of sunscreens against natural sunlight and UV-induced genotoxicity in Drosophila somatic cells. We prepared three kinds of sunscreen samples, each with an SPF value of 20, 40 or 60, and compared their protective activities with commercial sunscreens. When a sunscreen of SPF 20, 40 or 60 was pasted on the plastic cover of a petri dish in which Drosophila larvae were exposed to the sun or UV lamps, genotoxicity decreased as the SPF of the sunscreen increased, relative to levels of genotoxicity observed in samples without sunscreen. However, the protective abilities of sunscreens were unexpectedly not so different from each other. To reveal the relationship between the protective activity of sunscreen and the wavelength of light with which larvae were irradiated through the sunscreen, we measured the transmittance of light through the petri dish cover on which the sunscreen was pasted. Effective protection was demonstrated by removing components of light whose wavelengths were below 315 nm. We suggest that the measurement of anti-genotoxic activity and the determination of the wavelengths of light transmitted through the sunscreen should be an alternative method for evaluating the effectiveness of a sunscreen.

1. Introduction

Sunlight is indispensable for organisms living on earth. On the other hand, sunlight is biologically harmful. Numerous epidemiological investigations show that sunlight is carcinogenic to humans [1, 2], and the IARC classifies sunlight within Group 1, which includes human carcinogens [3].

The use of sunscreen seems to be important in avoiding damage caused by sunlight. The IARC has concluded that sunscreens reduced the risk of sunburn and probably prevent squamous cell carcinoma of the skin when used during unintentional sun exposure [4]. The application of sunscreen on skin reduces the risk of squamous cell carcinoma and basal cell carcinoma [5, 6]. Other investigations demonstrate the ability of sunscreen to protect against p53 responses and DNA photodamage [7, 8]. The biological activity of sunscreen, as represented by an SPF (Sun Protection Factor), is evaluated by its ability to protect human skin from erythema and edema. However, sunlight triggers many biological processes such as photoaging, immunosuppression, and mutation of skin cells. It has recently been suggested that the SPF value may not be a sufficient gauge of a sunscreen's ability to protect against the many harmful biological reactions induced by sunlight. Gill and Kim proposed the Immune Protection Factor (IPF) as a measure of the effectiveness of a sunscreen to protect against UV-induced immune suppression [9]. Ananthaswamy et al. proposed the Mutation Protection Factor (MPF) as an estimate of a sunscreen's protective activity, as obtained through the measurement of p53 mutation in the skin of mice irradiated with UVB [10]. It has also been shown that treatment with sunscreen reduced the incidence of tumors in the skin of mice irradiated by a solar simulator [11]. We also suggest that a sunscreen's ability to protect against sunlight-induced mutation should be taken into account. We have previously reported that sunlight was mutagenic in Drosophila somatic cells [12]. Many experiments in photobiology were invariably performed using artificial UV-light or a solar simulator, and did not measure the wavelength of light transmitted through the sunscreen. In this study we examine the protective activity of sunscreen against natural sunlight-induced

genotoxicity, compare the effectiveness of a sunscreen with its ability to block the passage of UV light, and discuss the relationship between protection against genotoxicity and SPF values.

2. Materials and Methods

2.1. Reagents

SPF 20 and 40 sunscreens were prepared by mixing three UV-absorbers (octyl methoxycinnamate [54466-7-3], 4-*t*-butyl-4'-methoxydibenzoylmethane [70356-09-1] and 2-hydroxy-4-methoxybenzophenone [131-57-7]) and one UV scattering agent (titanium oxide [13463-67-7]). SPF 60 sunscreen consisted of octyl methoxycinnamate, 4-*t*-butyl-4'-methoxydibenzoylmethane, titanium oxide and zinc oxide [1314-13-2]. Figure 1 shows the absorption spectra of a 0.1 mg/ml sunscreen solution in liquid paraffin and the transmittance of light through the plastic petri dish cover on which the sunscreen was pasted. Commercial sunscreens were purchased from drug stores in Okayama city. Absorption ointment was purchased from Merk (Darmstadt, Germany), and 2-(2-benzotriazolyl)-*p*-cresol [2440-22-4] and 4-dimethylaminobenzoic acid 2-ethylhexyl ester [21245-02-3] were from Tokyo Kasei Kogyo (Tokyo, Japan). The absorption spectra of these products in a 4 mg/ml DMSO solution are shown in Figure 2.

2.2. Drosophila strains

All Drosophila strains were provided as gifts by Dr. H. Ryo (Osaka University, Suita, Japan) and Dr. K. Fujikawa (Kinki University, Higashi-Osaka, Japan). The *in vivo* DNA repair test used the *sc* z_1 $w^{+(TE)}$ *mei-9*^{a} *mei-41*^{D5}*/C(1)DX, y f* strain, which consisted of DNA repair-deficient males and repair-proficient females. Two stocks, *y; mwh j v* for female and *y; Dp(1;3)sc*^{J4}, y^{+} *flr/TM1, Mé ri sbd*^{2} for male, were used in the Drosophila wing spot test to obtain the offspring for the somatic mutation assay. A recessive wing hair marker gene, represented by *mwh* and *flr*, is located on the third chromosome of each strain. These genotypes are described by Lindsley and Zimm [13].

2.3. Exposure to the sun and genotoxicity test

Third instar larvae were exposed to the sun using an apparatus as shown in Figure 3, which was located on a field within the campus of Okayama University as described

previously [12]. Sunscreen experiments involved pasting the covers of petri dishes with 0.25 mg/cm2 of sunscreen, with the transmittance of UV light being measured at 3 to 4 points for each cover. UVA- and UVB-doses were recorded by each sensor (a 365 nm peak-sensor for UVA and a 310 nm peak-sensor for UVB) connected to a UVX Radiometer (Uvp, Inc., Upland, CA). MED (Minimal Erythema Dose) was recorded using an Erythema UV Intensity & Dose Meter (Solar Light Co., Philadelphia, PA). Our observations in Okayama indicate that 1 MED is shown when UVA and UVB doses are 23 kJ and 7 kJ respectively. Larvae were transferred onto Drosophila instant medium (Formula 4-24, Carolina Biological Supply, Burlington, NC) after irradiation. This process was performed under a yellow lamp to avoid the possibility of photorepair. The larvae were kept in the dark at 25°C until adult flies emerged, after which counts were made of the number of male and female flies. The extent of DNA damage in the *in vivo* DNA repair test is shown by the sex ratio of repair-deficient males to repair-proficient females [14]. Somatic cell mutation was detected by counting spots possessing mutant wing-hairs [14, 15]. Statistical analysis was performed according to Frei and Würgler [16] and Kastenbaum and Bowman [17].

2.4. Exposure to fluorescent lamp

Larvae were irradiated with polychromatic UV in the manner reported previously [18]. Four fluorescent lamps (FL 20S · E, Toshiba, Tokyo) were used for UVB-irradiation (300-400 nm), and four black light lamps (FL 20S · BL-B, National, Tokyo) for UVA-irradiation (320 –400 nm). The UV doses on the larvae were 6 W/m2 for UVB trials and 4.4 W/m2 for UVA trials. The wing spot test and *in vivo* DNA repair test were performed as described above. In the in vivo DNA repair test, the UVB absorber was resolved in an ointment to 2.4%, 20% and 56%. In the wing spot test, UV-absorbers were resolved in liquid paraffin which has no absorbance of wavelengths below 400 nm.

3. Results

3.1. Effects of sunscreens against sunlight-induced DNA damage

Repairable DNA damage by sunlight was detected using the *in vivo* DNA repair test. Larvae consisting of repair-deficient males and repair-proficient females were exposed to the sun. Data representing 6 experiments, performed from March to November, is summarized in Figure 4. The weather on days on which an experiment was performed varied from sunny to cloudy. Levels of repair-deficient males hatching from pupae decreased as levels of UV fluence increased. A 10 kJ UVB-equivalent dose of sunlight, accumulated by insolation within 30 min on a sunny day, reduced the survival of repair-deficient males to 1%. The UVB dose required to reduce the survival of repair-deficient males to 1% rose to 50 kJ for tests involving SPF 20 sunscreen, 70 kJ for tests involving SPF 40 sunscreen, and 80 kJ for tests involving SPF 60 sunscreen. It is notable that even a sunscreen with an SPF value of 20 displayed effective protection against DNA damage.

3.2. Effects of sunscreens against sunlight-induced mutagenicity

Sunlight-induced mutagenicity was detected using the wing spot test. As shown in Table 1, mutation increased with an increase in the exposure time, except for the level of mutation observed from a 6hr exposure on a sunny day. The mutation was higher on a sunny day (Exp. 2) than on a cloudy day (Exp. 1), and was effectively suppressed by treatment with any of the tested sunscreens. Insolation from 10 a.m. to 2 p.m. on a sunny day was two-fold stronger than that observed on a cloudy day. When considering an exposure period of 2 hours, sunscreens with a high SPF value (40, 60) were more effective at suppressing mutation than sunscreens with a low SPF value (20), but there was no significant difference when the period of exposure rose to 4 hours. The survival of larvae decreased to 17% when exposed for 6 hours on a sunny day. However, treatment with a sunscreen protected the larvae from the lethal effects of sunlight, with survival rates after an exposure period of 6 hours being 60%, 50% and 70% for

sunscreen tests involving SPF 20, SPF 40 and SPF 60 respectively. We compared the protective efficacy of commercial sunscreens to that of sunscreen prepared in this study. Commercial SPF 40 sunscreen tested in this study only contains UV-scattering agents, such as zinc oxide and titanium oxide. As shown in Figure 5 (A), mutation induced by sunlight was suppressed to a similar extent when using either commercial or laboratory-prepared sunscreens. The transmittance curves shown by both sunscreens are also similar (Figure 1 (B) and Figure 5 (B)). We have obtained similar results in experiments using SPF 20 sunscreens. These results indicate that sunscreens defined by an identical SPF value seem to offer similar protection against mutation, despite possessing different ingredients if their transmittance should be similar.

3.3 Effects of sunscreens on UVA or UVB-induced mutagenicity

To examine protection against UVB or UVA-induced mutation, larvae were irradiated using fluorescent or black lamps in trials involving the presence or absence of sunscreen. As shown in Table 2, mutation induced by either UVA or UVB was effectively suppressed by treatment with each sunscreen. Although UVB 20 kJ/m2 is a lethal dose for Drosophila larvae (0 % survival), treatment with a sunscreen resulted in increased larval survival and decreased mutation. In contrast to low UVB doses, the protective abilities of sunscreens at high doses of UVB showed variation. SPF 40 and SPF 60 sunscreens almost completely suppressed mutagenicity, while the SPF 20 sunscreen did not suppress mutagenicity. As shown in Figure 1 (B), SPF 40 and SPF 60 sunscreens blocked wavelengths of light shorter than 320 nm. We suggest that effective protection, even at high doses of UVB, may occur by the avoidance of UV light with wavelengths shorter than 320 nm.

3.4. Effects of UV-absorbers on repairable DNA damage and mutation

To examine the protective activity of a single component when used as the active ingredient of a sunscreen, we prepared by mixing with ointment at various concentrations sunscreen containing only 2-(2-benzotriazolyl)-*p*-cresol for UVA-absorber trials, or 4-

dimethylaminobenzoic acid 2-ethylhexyl ester for UVB-absorber trials. The structures of these absorbers and their UV absorbance curves are shown in Figure 2. The UVB absorber trials show excellent protection against genotoxicity only for tests involving a high concentration (Figure 6, A-1). Transmittance of light through the plastic cover (Figure 6 A-2) shows that effective protection involved the sunscreen blocking wavelengths of UV light shorter than 315 nm. These results are consistent with the results of our initial sunscreen samples (Figure 1, 4). In contrast, UVA absorber trials showed no protective effect on the genotoxicity of UVB, even at the highest concentration (data not shown), whereas these trials displayed absorption within the UVB region (Figure 2A). Genotoxicity of UVA from black lamps is not detectable using the *in vivo* DNA repair test. The wing spot test was used to examine the effect of UV-absorbers on UVA mutagenicity. As shown in Figure 6 (B-1), a UVA-absorber resolved in paraffin is more effective in protecting against UVA-induced mutation than the UVB-absorber. The transmittance of wavelengths of light around 340 nm through plastic covers pasted with a UVA-absorber decreases to half of that observed for trials involving a UVB-absorber (Figure 6 (B-2)). The significant difference between the protective ability of UVA and UVB absorbers is less than 1 % when performing a X square test analysis.

4. Discussion

Although the use of sunscreen to avoid photodamage in human skin is a contentious issue [19], the general consensus seems to be that sunscreen could protect skin from photoaging, dermatitis or tumorigenesis induced by solar radiation [20]. Photoprotection is an especially important concept when considering the depletion of the ozone layer [21]. To date, the biological activity of sunscreens in offering protection against erythema has been represented through the use of a Sun Protection Factor (SPF). Some investigators have recently shown that SPF is not an adequate gauge when evaluating a sunscreen's ability to protect against UV-induced biological activity, and they propose estimating the protective activity of sunscreen through the use of other categories [10, 11, 22-24]. In this study we examined Drosophila systems to determine if a genotoxicity protection factor could be utilized as an alternative evaluation of the efficacy of a sunscreen. We pasted sunscreen samples on the covers of petri dishes, in which Drosophila larvae were exposed to the sun or irradiated by UV lamp. We determined the biological protective activity against photodamage using the *in vivo* DNA repair test and the wing spot test. The sunscreen's ability to block light was investigated by measuring the transmittance of light through the petri dish cover on which the sunscreen was pasted. The protection activity of these sunscreens against genotoxicity increased with an increase in SPF value. As the differences observed in protective activity against repairable DNA damage were smaller than that expected from SPF values, we propose obtaining a DNA Damage Protection Factor (DPF) from the slope of males surviving in sunscreen tests divided by that obtained in tests without sunscreen (Figure 4). DPF values of 43, 53, and 64 are proposed for sunscreens with SPF values of 20, 40 and 60 respectively. By using sunscreen with components similar to ours, Horiki et al. demonstrated that SPF 60 sunscreen was more effective than SPF 10 sunscreen in protecting against photocarcinogenesis, photoaging, and formation of cyclobutane pyrimidine dimers induced by UVB-irradiation in XPA gene knockout mice [25]. Our results suggest that effective protection against UVB-genotoxicity might be gained by the avoidance of light

with wavelengths shorter than 315 nm. Young et al. reported that the formation of pyrimidine dimers was inhibited by UVA and UVB sunscreen to similar degrees, and led to the inhibition of erythema in human skin in experiments using solar-simulated radiation [26]. A UVA absorber in our study did not reduce the lethality of repair-deficient Drosophila from UVB-genotoxicity, but the UVB absorber displayed good protection. In contrast, the UVA-absorber is more effective in protecting larvae from UVA-mutagenicity than the UVB-absorber. The discrepancy between our results and those of Young et al. appears to be due to the absorbers used in each study. The effect of a sunscreen is influenced by the manner of its application, such as doses and times, and an SPF value alone may not reflect the efficacy of protection possessed by that sunscreen [27]. It is difficult using human skin to evaluate the biological activity of sunscreen in a uniform manner. We suggest that the anti-DNA damaging activity of a sunscreen, by mechanical coating, may be an alternative indicator for evaluating the efficacy of a sunscreen. Moreover, we could estimate the biological protective efficacy of a sunscreen by measuring the wavelengths of light transmitted through the sunscreen.

The possibility exists that UV-absorbance by the sunscreen may change after exposure to UV light. We observed significant increases in transmittance for SPF 20 trials during a 2 hour period of exposure to the sun, but this phenomenon was not observed for SPF 40 trials (data not shown). Photo-inactivation of sunscreens may be responsible for the underestimation of UV-damage as described by Maier et al [28]. We have observed the advantages of repeated application of sunscreen during a period of 4 hours of solar radiation (data not shown). To avoid damage caused by prolonged exposure to the sun, the effective utilization of sunscreen should be further encouraged. On the other hand, Xu and Parsons have reported the cytotoxic effect of a sunscreen component in cultured human cells [29], although Dean et al showed no photomutagenicity of three sunscreen ingredients with UV light using bacterial reverse mutation and a mammalian chromosome aberration assay [30]. The Drosophila systems used in this study could also be used to examine the genotoxicity of the sunscreen itself, with an investigation of the effects of direct contact of sunscreen with larvae being one approach. Further research is required,

with a possible shift in paradigms that estimate the protective efficacy of a sunscreen, in an effort to yield sunscreens that better reduce the biologically harmful effects of sunlight to humans.

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Table 1 Protective effect of sunscreens against sunlight-induced mutagenicity

exposure time (hr)	UVA (kJ/m2)	UVB (kJ/m2)	sunscreen	survival (%)	No. of wings	Total spots	Total spots/ wing	Inhibition (%) *a)*
Exp. 1 (18, April; cloudy)								
dark	0	0	-	100	200	70	0.35	-
2	32	9	none*b)*	97	100	80	0.80	0
			20	99	100	56	0.56	53
			40	95	100	43	0.43	82
			60	83	100	28	0.28	100
4	59	16	none	75	99	170	1.72	0
			20	83	100	73	0.73	72
			40	88	100	68	0.68	76
			60	75	93	59	0.63	80
Exp. 2 (19, April; sunny)								
dark	0	0	-	100	200	79	0.40	-
1	35	9	none	100	86	93	1.08	0
			20	98	98	58	0.59	72
			40	113	84	55	0.66	63
			60	87	96	51	0.53	89
2	74	20	none	82	65	93	1.43	0
			20	98	96	79	0.82	59
			40	105	81	48	0.59	82
			60	93	76	42	0.55	81
3	113	30	none	78	54	93	1.72	0
			20	90	78	77	0.98	56
			40	85	94	100	1.06	50
			60	103	84	54	0.64	82
4	147	40	none	74	45	86	1.91	0
			20	74	59	56	0.95	64
			40	80	60	52	0.86	69
			60	66	53	36	0.68	81
6	193	51	none	17	33	45	1.36	0
			20	59	38	57	1.50	-14
			40	50	44	39	0.89	49
			60	69	39	26	0.67	72

a) Inhibition percent (%) = 100 - (spots/wing in the exposure with sunscreen - spots/wing in the dark)/ (spots/wing in the exposure without sunscreen - spots/wing in tha dark) x 100
b) none: without sunscreen

Table 2 Protective effect of sunscreens against UV-induced mutagenicity

UV dose kJ/m2	sunscreen	survival %	No. of wings	Total spots	Total spots/ wing	Inhibition %*a)*
UVB						
0	-	100	152	40	0.26	-
10	none*b)*	72	135	211	1.56	0
	40	104	100	40	0.40	89
	60	75	43	21	0.49	82
20	none	0	0	-	-	-
	20	100	161	156	0.96	-
	40	95	117	61	0.52	-
	60	92	138	50	0.36	-
UVA						
0	-	100	206	163	0.56	-
250	none	78	102	117	1.11	0
	20	89	121	114	0.94	13
	40	116	112	78	0.70	77
	60	78	134	95	0.71	89
300	none	91	68	102	1.50	0
	20	108	76	60	0.79	74
	40	108	101	64	0.63	91
	60	96	80	50	0.63	91

a) Inhibition percent (%) = 100 - (spots/wing in the exposure with sunscreen - spots/wing in the dark)/ (spots/wing in the exposure without sunscreen - spots/wing in the dark) x 100
b) none: without sunscreen

Figure legends

Figure 1 The absorbance of a 0.1 mg/ml sunscreen solution in liquid paraffin (A) and transmittance through the cover of a plastic petri dish on which was pasted 0.25 mg/cm2 of sunscreen (B).

Figure 2 The absorption spectra of 2-(2-benzotriazolyl)-*p*-cresol (A) and 4-dimethylaminobenzoic acid 2-ethylhexyl ester (B). The concentration of each solution is 4 mg/ml in dimethyl-sulfoxide.

Figure 3 Apparatus used for trials involving exposure to the sun.

Figure 4 Effects of sunscreens on sunlight-induced repairable DNA damage. Third instar larvae were exposed to the sun in petri dishes whose covers were pasted with 0.25 mg/cm2 of each sunscreen (●), or not (◯), on March (cloudy), April (sunny), May (sunny), September (cloudy after sunny, sunny and sometimes cloudy) and November (cloudy and sometimes sunny). UV fluence was measured as described in "Materials and Methods". The sex ratios without exposure varied from 0.7 to 1.46 at each experiment. To normalize data in each experiment, genotoxicity is shown by surviving fractions, which are obtained after dividing the sex ratio with exposure by the sex ratio without exposure.

Figure 5 Suppressive effects on sunlight-induced mutation by sunscreens with identical SPF values. (A) The detection of mutation, and the manner of treatment with SPF 40 sunscreen, was as described in "Materials and Methods". Without sunscreens: ■, with the sunscreen prepared by ourselves: ▭, with the commercial sunscreen: ▭, and no exposure: ▭. (B) Transmittance through the cover of the petri dish on which was pasted 0.25 mg/cm2 of commercial sunscreen.

Figure 6 The effects of a UV-absorber on genotoxicity induced by UV -irradiation. (A-1): Protection of UVB absorber against UVB-genotoxicity shown by an open circle for no sunscreen, and using a 20% absorber (closed triangle) and 56% absorber (closed circle) relative to no protection with a 2.4% absorber (closed square). (B-1): Protection of a UV-absorber against UVA-induced mutagenicity. Mutagenicity is shown by total spots/wing with no sunscreen (), by pasting paraffin used as a solvent of absorber (), pasting a 20 % UVA absorber (), pasting a 20 % UVB absorber (), and control without irradiation (). The transmittance of each sunscreen sample is shown in (A-2) and (B-2).

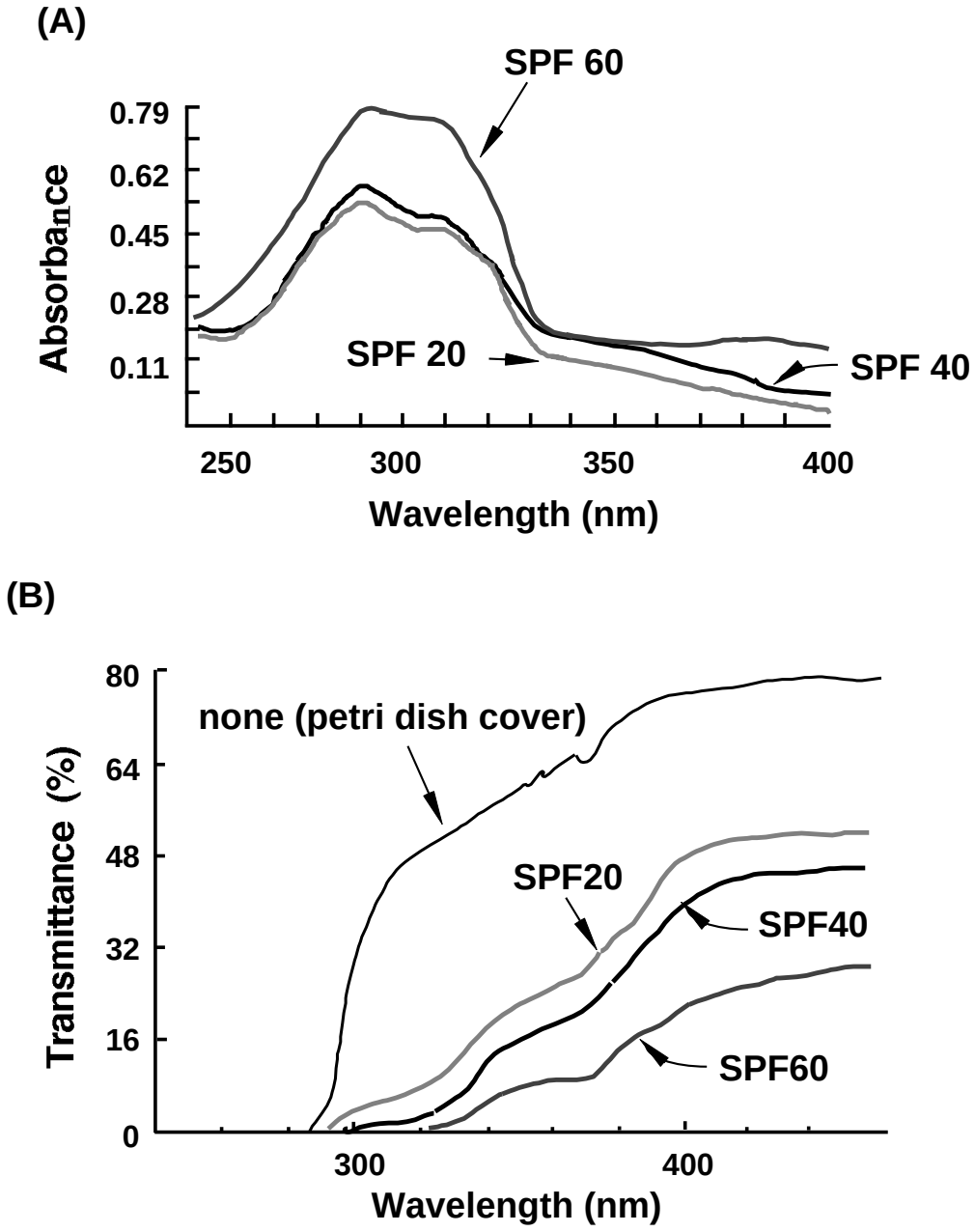
(A)
SPF 60
0.79
0.62
0.45
0.28
0.11
Absorbance
SPF 20
SPF 40
250
300
350
400
Wavelength (nm)
(B)
80
64
48
32
16
0
Transmittance (%)
none (petri dish cover)
SPF20
SPF40
SPF60
300
400
Wavelength (nm)

Figure 1

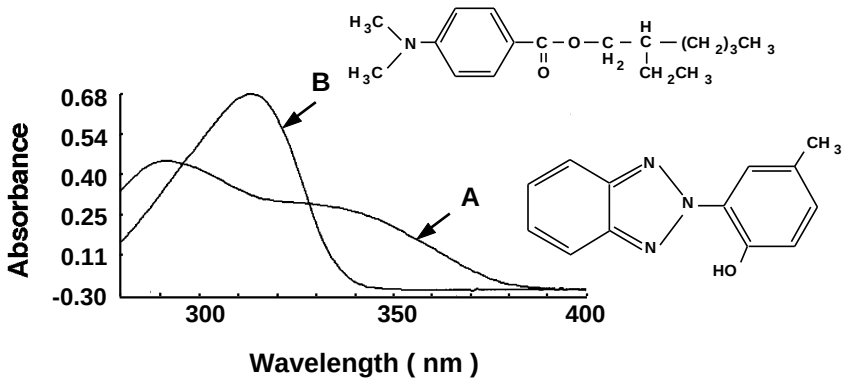
B
A
Absorbance
0.68
0.54
0.40
0.25
0.11
-0.30
300
350
400
Wavelength (nm)
HO

Figure 2

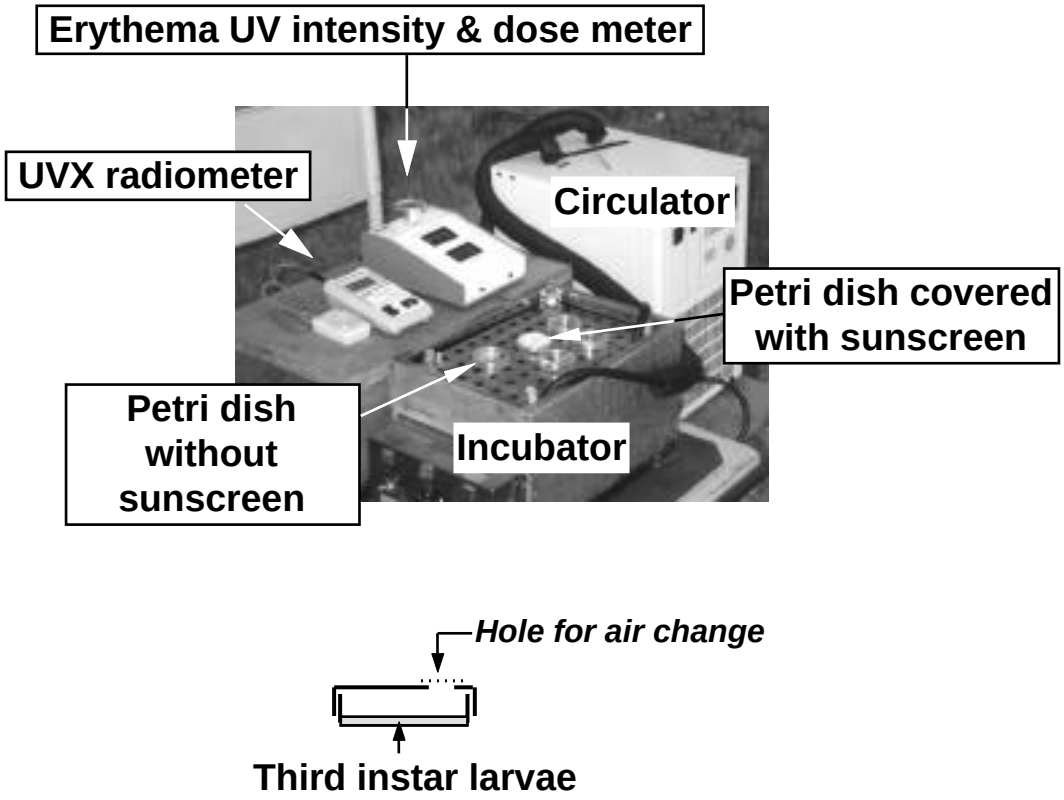
Erythema UV intensity & dose meter
UVX radiometer
Circulator
Petri dish covered with sunscreen
Petri dish without sunscreen
Incubator
Hole for air change
Third instar larvae

Figure 3

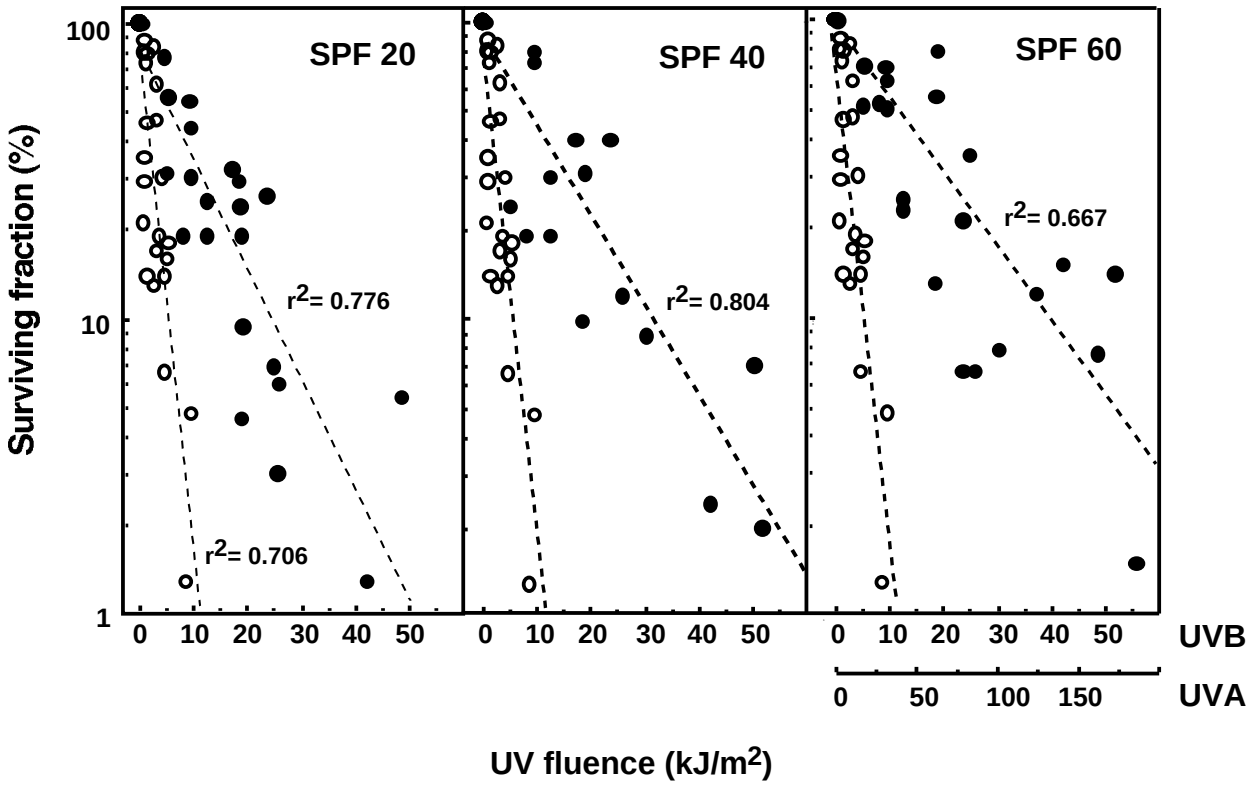
SPF 20
SPF 40
SPF 60
Surviving fraction (%)
100
10
1
r2= 0.776
r2= 0.706
r2= 0.804
r2= 0.667
0 10 20 30 40 50
UVB
0 50 100 150
UVA
UV fluence (kJ/m2)

Figure 4

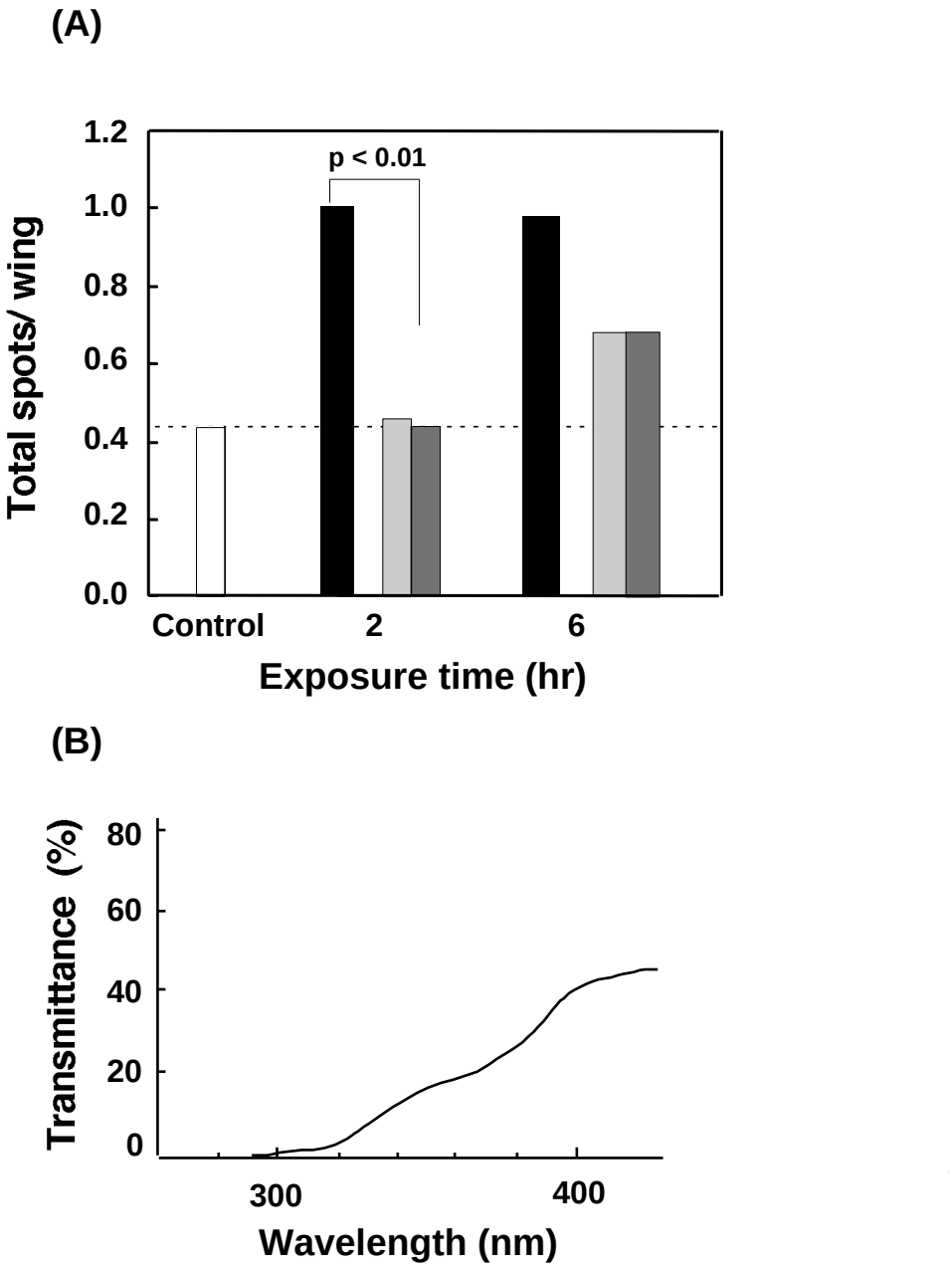
(A)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
p < 0.01
Total spots/ wing
Control
2
6
Exposure time (hr)
(B)
80
60
40
20
0
Transmittance (%)
300
400
Wavelength (nm)

Figure 5

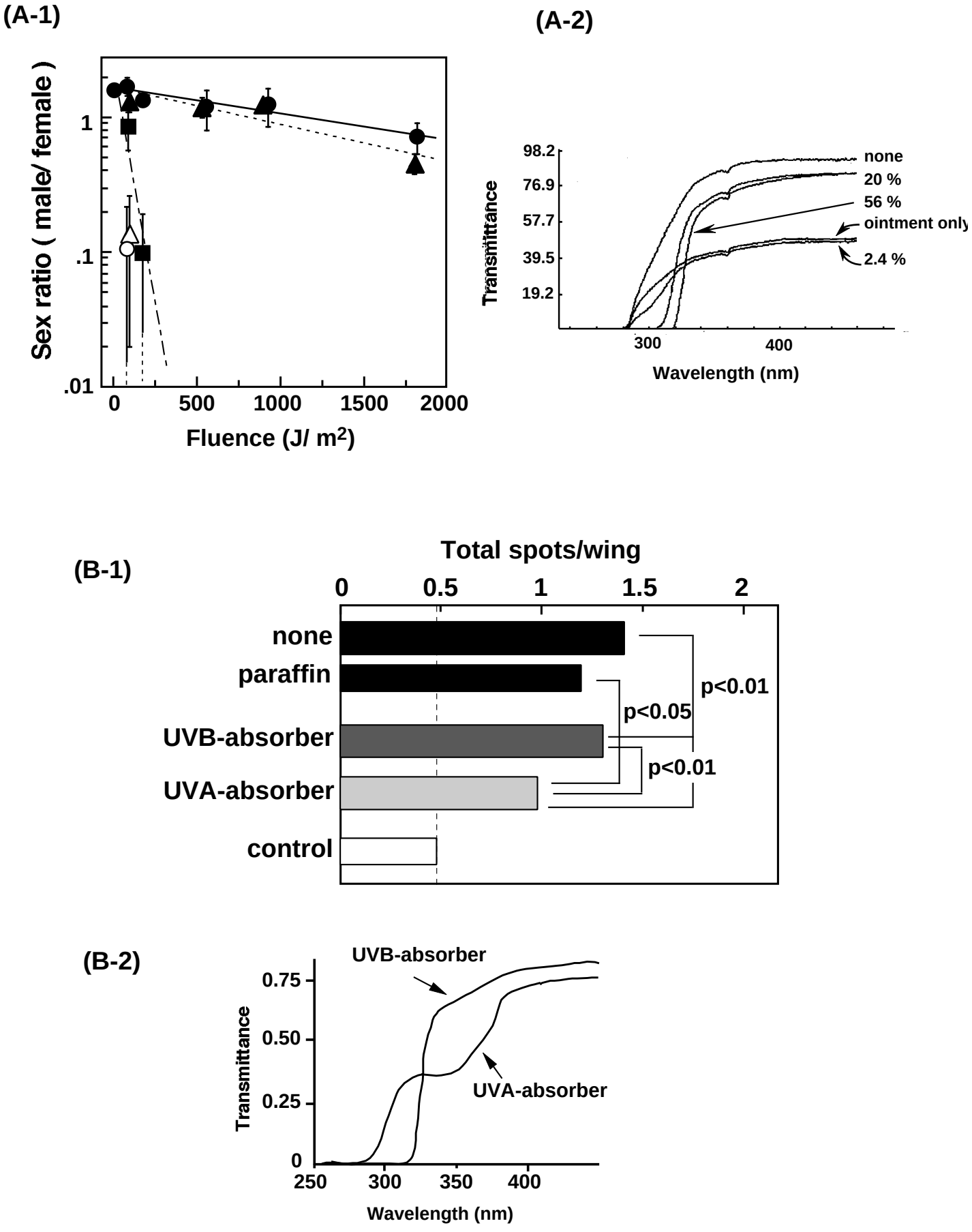
(A-1)
Sex ratio (male/ female)
1
.1
.01
0
500
1000
1500
2000
Fluence (J/ m2)
(A-2)
Transmittance
98.2
76.9
57.7
39.5
19.2
300
400
Wavelength (nm)
none
20 %
56 %
ointment only
2.4 %
(B-1)
Total spots/wing
0
0.5
1
1.5
2
none
paraffin
UVB-absorber
UVA-absorber
control
p<0.01
p<0.05
p<0.01
(B-2)
UVB-absorber
UVA-absorber
Transmittance
0.75
0.50
0.25
0
250
300
350
400
Wavelength (nm)

Figure 6