Phototoxicity of Melatonin

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Melatonin (MLT), N-acetyl-5-methoxytryptamine, is mainly secreted by the pineal gland. The ultraviolet (UV), infrared (IR) and ^{1}H-NMR spectra of irradiated and non-irradiated MLT were measured, and phototoxicity tests of MLT, anthracene (positive control) and sodium lauryl sulfate (SLS, negative control) were performed. The methods employed include both *in vitro* tests such as MTS assay using the human fibroblast cell and yeast growth inhibition assay using *Candida albicans* and *in vivo* method using the skin of guinea pig. UV absorption spectra and ^{1}H-NMR spectra of MLT were changed by UVA (365 nm, 15 J/cm^2), but IR spectra of MLT were not changed. The fifty percent inhibitory concentration (IC_{50}) ratio (UV-/UV+) of MLT was 10. The inhibition zone of irradiated-paper disks treated with MLT was not observed. According to the results of histopathological examination, no pathologic lesion was observed in the non-irradiated group, but slight degeneration of keratinocytes in the epidermis, hemorrhage and vasodilation in dermis were observed in the irradiated group. These results indicate that the molecular structure of MLT is altered by UVA to unidentified photoproducts and a moderate phototoxicity of MLT is predicted.

Key words : Melatonin, Phototoxicity, Yeast growth inhibition

INTRODUCTION

Phototoxicity is here defined as toxic responses that are elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or those that are induced similarly by skin irradiation after systemic administration of a chemical substance into body. This is to be distinguished from photoallergy in so far as the latter involves the immunological system (Maurner, 1987; Cronin, 1980; Gabriel *et al.*, 1985; Marzulli *et al.*, 1987). The primary reactions responsible for initiating phototoxicity can often be described in terms of basic photochemical reaction patterns, often involving molecular oxygen (photodynamic action) with or without generation of singlet state excited oxygen (Kramer *et al.*, 1972; Nilsson *et al.*, 1972, 1975). Prolonged exposure to the sunlight in the 280~315 nm range (UVB) has traditionally been held responsible for up to 95% of human skin cancers other than malignant melanomas, since exposure to these wavelength can produce carcinogenesis in hairless mice (Staberg *et al.*, 1983) and mutation in bacteria (Peak *et al.*, 1984). On the other hand, Melatonin (MLT) is mainly secreted by the pineal gland into blood circulation in most animal species and has several roles as a hormone (Reiter, 1991). The pineal gland functions as a neuroendocrine transducer, receiving light information from retina and transforming this neural signal into chemical messengers, the best known of which is MLT (Axelrod *et al.*, 1982). This hormone is synthesized from tryptophan, which is initially hydroxylated and then decarboxylated giving serotonin (via N-acetyltransferase, NAT) and subsequent methylation (via hydroxyindol-o-methyltransferase) yielding MLT. Circadian rhythms in the pineal indole metabolism, especially MLT, are regulated primarily by the activity of the enzyme NAT (Ssavedra *et al.*, 1973). Being aware of the rapid percutaneous penetration of MLT into human skin, since MLT, which is not metabolized in human skin (Lee *et al.*, 1994), does not absorb UVA or UVB, a direct sunscreen effect of this substance can be excluded. However, the UVB-sensitized tryptophan, a precusor of MLT, produces singlet oxygens and superoxide radicals, and these reactive forms of oxygen may contribute to membrane-, cytoplasm- and DNA-damaging effects (Baau *et al.*, 1992). The purpose of this study is to manifest that whether the photoproducts produced by photolysis of

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MLT can absorb UVA or not, and phototoxicity is induced by the photoproducts or not. In this study, we have identified the degradation of MLT with UVA through the measurement of the UV, IR and ^{1}H-NMR spectra, and UV absorption of the reaction products obtained from photolysis of MLT, finally, phototoxicity of MLT was predicted.

MATERIALS AND METHODS

Chemicals

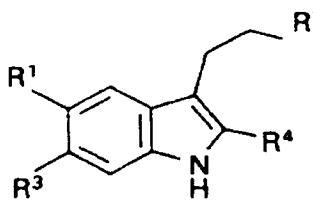
MLT (Fig. 1), anthracene (positive control), sodium lauryl sulfate (SLS, negative control) and MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] used in this study were purchased from Sigma Chemical Co. (St. Leuis, MO, USA). Dimethylsulfoxide (DMSO) was provided from Wako pure Chemical Co. (Osaka, Japan).

Animals

Albino Hartley strain guinea pigs weighing 300~400 g (3~4 weeks old, male) were used. All animals were fed pelleted diet and water *ad libitum* with daily supplements of hay and cabbage. Hair was removed by clipping and shaving.

Equipment

Ultraviolet (UV) Visible (Vis) spectophotometer was purchased from Hewlett Packard (Sanfrancisco, CA, USA), and Infrared (IR) spectophotometer was supplied by Hewlett Packard, and Nuclear magnetic resonance (NMR) was provided from JOL Co. (Japan), and ELISA was purchased from Molecular Devices Co. (USA).



Compound	R^1	R^2	R^3	R^4
1. 2-Chloromelatonin	OMe	NHCOMe	H	Cl
2. Melatonin	OMe	NHCOMe	H	H
3. 6-Fluoromelatonin	OMe	NHCOMe	F	H
4. 6-Chloromelatonin	OMe	NHCOMe	Cl	H
5. 6-Hydroxymelatonin	OMe	NHCOMe	OH	H
6. N-isobutanoyl 5-methoxytryptamine	OMe	NHCOPr	H	H
7. N-valeroyl 5-methoxytryptamine	OMe	NHCOBu	H	H
8. 6-methoxymelatonin	OMe	NHCOMe	OMe	H
9. 5-methyl N-acetyltryptamine	Me	NHCOMe	H	H
10. 5-Benzoyl N-acetyltryptamine	OCH_2ph	NHCOMe	H	H
11. O-acetyl 5-methoxytryptamine	OMe	OCOMe	H	H
12. N-acetyltryptamine	H	NHCOMe	H	H
13. N-acetyl 5-hyroxytryptamine	OH	NHCOMe	H	H
14. 5-Methoxytryptamine	OMe	NH	H	H

Fig. 1. Chemical structures of melatonin analogues.

Light source

The light source was a bank of 14 fluorescent black lights (Dong Sung Labtech Co., Korea) with an emission spectra of 200~430 nm, mainly UVA region peaking at 365 nm. The irradiance was measured by a radiometer (Vilver Lourmat, France).

UV absorption spectra

MLT, anthracene and SLS scattered on the petri dish, were irradiated with UV 365 nm (15 J/cm^2). Irradiated compounds and non-irradiated compounds were weighed, and dissolved in DMSO, and the optical density of each solution was measured by spectrophotometer set at 200~600 nm, respectively.

IR spectra

The IR spectra of the compounds were measured according to potassium bromide tablet method.

^{1}H-NMR spectra

^{1}H-NMR spectra of MLT, anthracene, SLS and irradiated compounds of those were measured in a proton NMR (400 MHz) using methanol-d_4 as solvent and tetramethylsilan as standard material.

MTS assay

MTS assay was performed according to the method described by Goodwin (1995). The cells used in this study were human fibroblast cells induced from foreskin. The skin tissue was transferred into the pertri dish, washed with phosphate buffered saline (PBS, Sigma Co., USA), and lipid was removed by scissors. After washing the cells three times with PBS and Dulbecco's Minimal Essential Medium (DMEM, Gibco, USA), collagenase (1 mg/ml, Gibco, USA) and dispase (2 mg/ml, Gibco, USA) were added and incubated at 37°C for 45 min. Cells were cultured in duplicate in 0.1~0.2 ml of appropriate growth medium in 96 well flat-bottomed culture plates at 1×10^5 cells/well concentration. After adding the samples into the cells, the plates were irradiated with 2.5 J/cm^2 of UVA (365 nm) and incubated at 37°C, 5% CO_2, for 1 hr. MTS/PMS solution (20 μl) was added into each well 20 hr after incubation. The amount of reduced formazan was assayed by measuring the optical density at 490 nm using ELISA plate reader (Molecular Device, USA) 3 hr after the addition of MTS solution. The IC_{50} was calculated using the method by Litchfield and Wilcoxon (1994), and the chemicals were evaluated as phototoxic positive if the differences in IC_{50} before and after

irradiation was 5 times and more.

Yeast growth inhibition assay

Yeast growth inhibition assay was conducted according to the method described by Ljunggen (Ljunggren *et al.* 1986) using *Candida albicans* ATCC 10231, Cells were cultured in Sabouraud's dextrose (SD) agar medium, taken by wire loop, and diluted with 5 ml sterile water. Diluted *Candida albicans* suspension was seeded on the SD agar plate by cotton lode. The paper disks absorbing the 30 μl of sample solution, were seeded on the SD agar plates after complete drying. The plates were exposed to 60 J/cm^2 of UVA (365 nm), and further incubated at 25°C for 72 hr after irradiation. The diameters of inhibition zones formed around the disks were measured 24, 48 and 72 hr after the incubation. The compounds formed an inhibition zone (of 2 mm diameter or above) 24 hr after incubation only in irradiated groups were evaluated as phototoxic positive.

In vivo phototoxicity test

The technique used in the in this study was basically the same as the phototoxicity test reported by Morikawa *et al.* (1974). Twenty-four hr prior to topical application of the materials, hairs on 6 cm×8 cm area on the back of the animal was clipped and shaved. Four groups of guinea pigs including the normal, positive control, negative control and test group, were used. MLT and SLS were weighed in a volumetric flask, and dissolved in DMSO, and diluted serially to 10, 1 and 0.1%. Anthracene which has dissolved by the same vehicle was diluted to 0.1, 0.01 and 0.001%. A volume of 15 μl/cm^2 was applied with a micropipette to each site measuring 1.5 cm×1.5 cm. The treated sites were placed in such a way to prevent the mixing of the test solutions. Immediately after the cutaneous application, the animals were placed in a restrainer, and the left site was irradiated with 15 J/cm^2 of UVA. Each test site, either irradiated or non-irradiated, was examined 24 hr and 48 hr after topical application of test solutions. The responses were scored blindly according to the following grading scale; 0= no reaction, 1=very slight erythema, 2=well-defined erythema, 3=moderate to severe erythema, 4=severe erythema. The average score of 5 guinea pigs was calculated, and assessed as phototoxic positive if the value was above 0.6.

Histopathological examination

All mice were sacrificed 48 hr after irradiation, and the skins of the treated sites were obtained, and histopathological examination was performed with the samples after fixing with 10% formalin.

RESULTS

Changes in UV, IR and ^{1}H-NMR spectra

UV spectra of MLT, anthracene and SLS were changed by UVA (Fig. 2). Especially, the significant differences in UV absorption spectra of anthracene were observed after irradiation, but not for the SLS. In case of MLT, the UV absorbance was not observed at 200~400 nm before irradiation, but strong UV absorbance was shown after irradiation. It was shown in Fig. 3 that IR spectra of all samples were not changed by UVA (365 nm). Fig. 4 shown which ^{1}H-NMR spectra of all samples except SLS were changed by UVA. On the other hand, the peaks below 6 ppm, which correspond to the absorbance of UVA, were changed after irradiation in treatment of MLT or anthracene.

MTS assay

Results of phototoxicity of MLT using MTS assay were shown in Table I and Fig. 5. IC_{50} of before and after irradiation were 200 and 20, respectively in treatment of MLT, The ratio of IC_{50} (UV-)/IC_{50} (UV+) was greater than 10, therefore MLT was evaluated as positive in phototoxicity compared to SLS (negative control).

Yeast growth inhibition test

The growth inhibition zone of irradiated-paper disks showed a dose-dependency (Table II, Fig. 6), but that

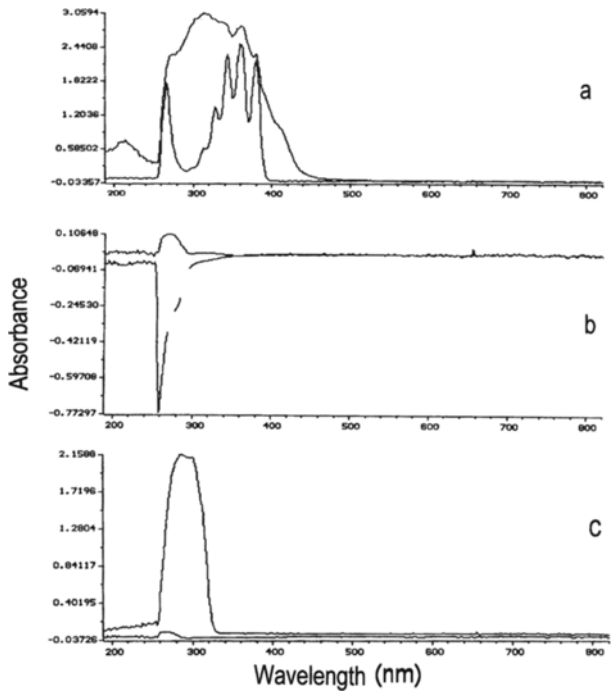


Fig. 2. Ultraviolet absorption spectra of the chemicals. a: anthracene, b: sodium lauryl sulfate, c: melatonin. Upper spectra: irradiation, lower spectra: non-irradiation.

of MLT was not changed at all. In the case of SLS, inhibition zone of both irradiated and non-irradiated groups was grown at higher concentration (10 w/v%). These results suggest that MLT does not induce the phototoxicity.

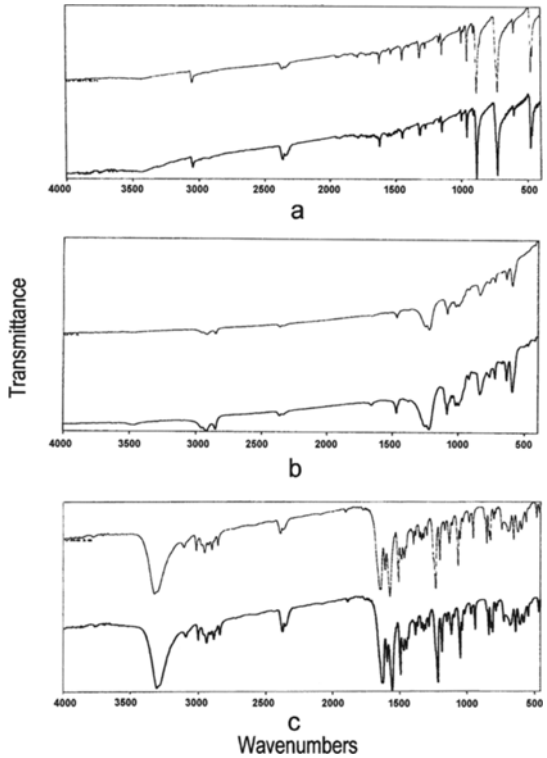


Fig. 3. IR absorption spectra of the chemicals. a: anthracene, b: sodium lauryl sulfate, c: melatonin. Upper spectra: irradiation, lower spectra: non-irradiation.

In vivo phototoxicity assay

The results of *in vivo* phototoxicity test using guinea pigs are shown in Table III. The phototoxicity of anthracene was observed in a dose- or time-dependent manner, and SLS did not induce the phototoxicity at all concentrations (10, 1, 0.1 w/v%) used. Phototoxicity of MLT was observed at two concentrations (10, 1 w/v%), but not at 0.1 w/v%, and phototoxicity was highest 24 hr after irradiation.

Table I. Phototoxicity of melatonin by MTS assay using human fibroblast cells

Chemicals	MTS assay			
	IC_{50} (μg/ml)		IC_{50} (UV-)/ IC_{50} (UV+)	Evaluation
	(UV-)	(UV+)		
Anthracene (positive control)	>15[NC]	0.038 ±0.005	>394.7	+
Sodium lauryl sulfate (negative control)	11.5 ±0.5	11.3 ±0.4	1	-
Melatonin	200[NC]	20 ±2	>10	+

[NC]: No cytotoxicity could be determined by yeast growth inhibition assay using *Candida albicans*.

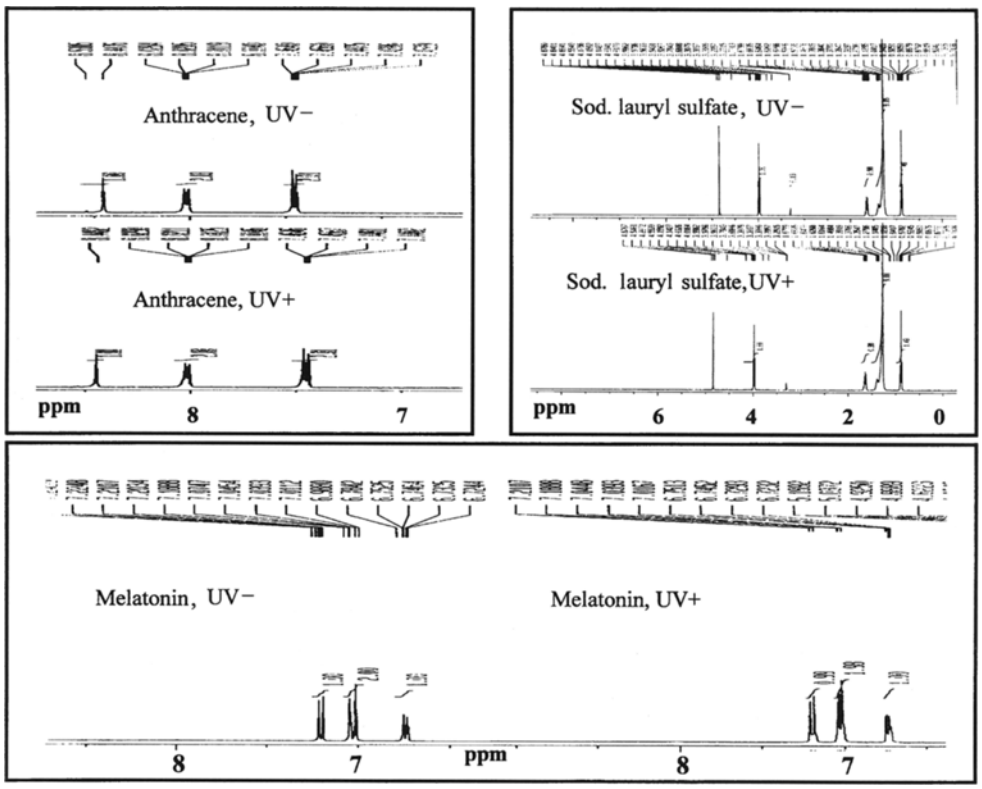


Fig. 4. ^{1}H-NMR spectra of the chemicals.

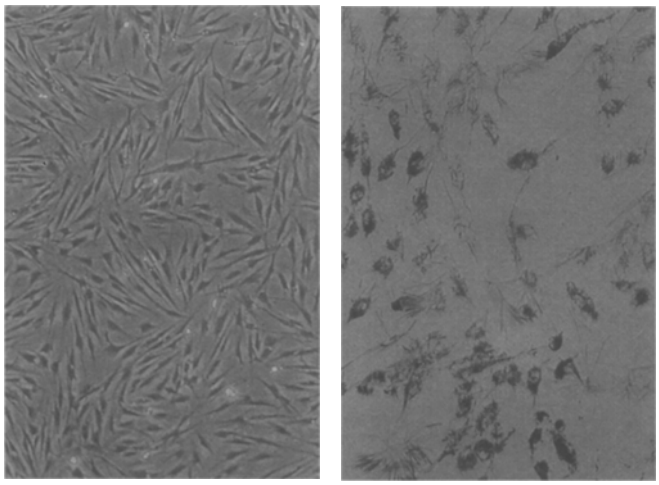


Fig. 5. Photomicrographs of human fibroblast cell culture exposed to melatonin in MTS assay. Left: non-irradiation, right: irradiation.

Table II. Dose-dependent phototoxicity of the several chemicals by yeast growth inhibition assay using *Candida albicans*

Chemicals	Conc. (%)	Growth Inhibition Zone (mm)	
		UV-	UV+
Anthracene (positive control)	0.1	0	4.83±0.76
	0.01	0	2.83±0.76
	0.001	0	2.00±0.00
Sodium lauryl sulfate (negative control)	10	3.00±1.00	3.00±1.00
	1	0	0
	0.1	0	0
Melatonin	10	0	0
	1	0	0
	0.1	0	0

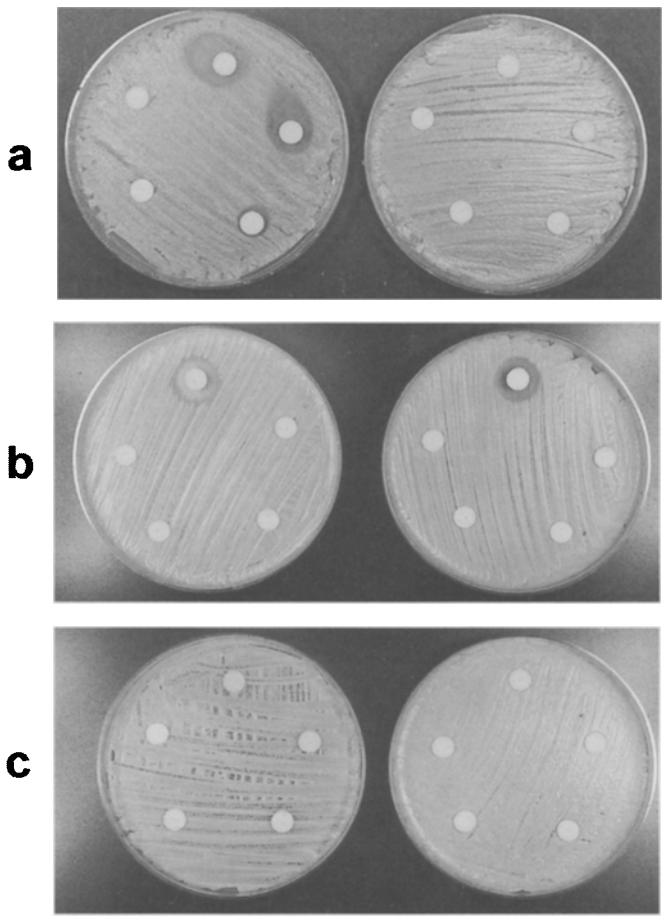


Fig. 6. Phototoxic responses of irradiated and non-irradiated chemicals in yeast inhibition test. a: anthracene, b: sodium lauryl sulfate, c: melatonin. Left: irradiation, right: non-irradiation.

Histopathological test

The results obtained from the present test were shown in Table IV and Fig. 7. Hypergranulosis was observed in the irradiated group which was treated with DMSO, and for the group which was treated with anthracene as a positive control, severe degeneration of epidermis and moderate cellular infilteration, vasodilation and edema were observed in the irradiated group, and slight hypergranulation was recognized in irradiated and non-irradiated group which was treated with SLS as a negative control. In the case of MLT, no pathologic lesion was observed in the non-treated group, but slight degeneration of keratinocytes in the epidermis, hemorrhage and vasodilation in dermis were observed in the irradiated group.

DISCUSSION

The purity (including significant impurities), solubility, characteristics, pH, stability (including photostability and stability in vehicle when so applied) of the test material should be assessed prior to experiment. Knowledge on the light absorption pattern may help design the experiment with respect to appropriate irradiation conditions and applied concentrations (Nilsson, 1993). MLT is dissolved in DMSO, and diluted to below 1% DMSO. The saturated solution of MLT in DMSO was selected as the highest dose, and other sample solutions were prepared in 10-fold dilution. UV absorption spectra and ^{1}H-NMR spectra of MLT were changed after irradiation, and especially the UV absorbance was increased significantly after irradiation. These results suggest that the photolysis of MLT was induced by UVA, and the photoproducts absorbed the UV in contrary to the result by Lee *et al.* (1994). Therefore, we could predict that phototoxic reaction was induced by topical treatment of MLT, as the majority of known phototoxic materials absorb the light in the UVA wavelengths. The two *in vitro* methods used in the present study have difference in the phototoxic mechanism. That is, the major reaction mechanism of yeast growth inhibition

Table III. Dose- or time-dependent phototoxicity of several chemicals by *in vivo* phototoxicity tests using guinea pigs

Chemicals	Conc. (%)	Time after Irradiation	Reaction Score	Reaction Rate	Mean Value
Anthracene (positive control)	0.1	12	3 2 2 1 2	5/5	2
		24	3 2 3 3 3	5/5	2.8
		48	4 3 3 2 3	5/5	3
	0.01	12	2 1 1 1 1	5/5	1.1
		24	2 2 2 1 1	5/5	1.6
		48	2 2 2 2 2	5/5	1.8
	0.001	12	1 0 1 0 0	2/5	0.4
		24	1 1 1 1 1	5/5	1
		48	1 1 1 1 0	4/5	0.8
Sodium lauryl sulfate (negative control)	10	12	0 0 0 1 0	1/5	0.2
		24	0 0 0 1 1	2/5	0.4
		48	0 0 0 1 0	1/5	0.2
	1	12	0 0 0 1 0	1/5	0.2
		24	0 0 0 1 0	1/5	0.2
		48	0 0 0 1 0	1/5	0.2
	0.1	12	0 0 0 0 0	0/5	0
		24	0 0 0 0 0	0/5	0
		48	0 0 0 0 0	0/5	0
Melatonin	10	12	1 1 1 1 0	4/5	0.8
		24	2 2 1 2 0	4/5	1.4
		48	1 1 2 1 1	4/5	1.2
	1	12	1 1 0 0 1	3/5	0.6
		24	1 1 0 1 1	4/5	0.8
		48	1 0 0 1 1	3/5	0.6
	0.1	12	0 0 0 0 0	0/5	0
		24	0 0 0 0 0	0/5	0
		48	0 0 0 0 0	0/5	0

Table IV. Histopathological changes in the skin tissues of irradiated or non-irradiated guinea pigs 48 hr after irradiation

	DMSO		Anthracene		SLS		MEL	
	UV-	UV+	UV-	UV+	UV-	UV+	UV-	UV+
Epidermis								
Thickening[a]	-	-	-	-	-	-	-	-
Degeneration	-	-	-	+++	-	-	-	+
Edema	-	-	-	-	-	-	-	-
Hypergranulation[b]	-	±	-	-	+	+	-	-
Microvesicles	-	-	-	-	-	-	-	-
Dermis								
Cellular infiltration	-	-	-	++	-	-	-	-
Vasodilation	-	-	-	++	-	-	-	+
Hemorrhage	-	-	-	-	-	-	-	+
Edema	-	-	-	++	-	-	-	-

Abbreviations: -, negative; ±, very slight; +, slight; ++, moderated; +++, marked. UV+, irradiation; UV-, non-irradiation.
[a]increased thickness of epidermal layers, including the stratum corneum.
[b]recognized by the darkblue (H & E) granules of keratohyalin in stratum corneum.

assay is the damage to DNA or cell organelles (Sugiyama *et al.*, 1994), and MTS assay has been used to detect cytotoxic or growth inhibitory lymphokines (Lora *et al.*, 1984). In this study, it is predicted that MLT caused the phototoxicity according to the results of MTS assay, but phototoxic reaction was not observed in yeast growth inhibition assay. These results indicate that MLT induce the phototoxicity to influence on cytotoxic or growth inhibitory lymphokines. Phototoxicity of MLT was enhanced in irradiated guinea pigs along with the increase of MLT dose, and it peaked 24 hr after irradiation. Consequently, it is predicted that MLT induce moderate phototoxicity compared with anthracene and SLS. The above results are sup-

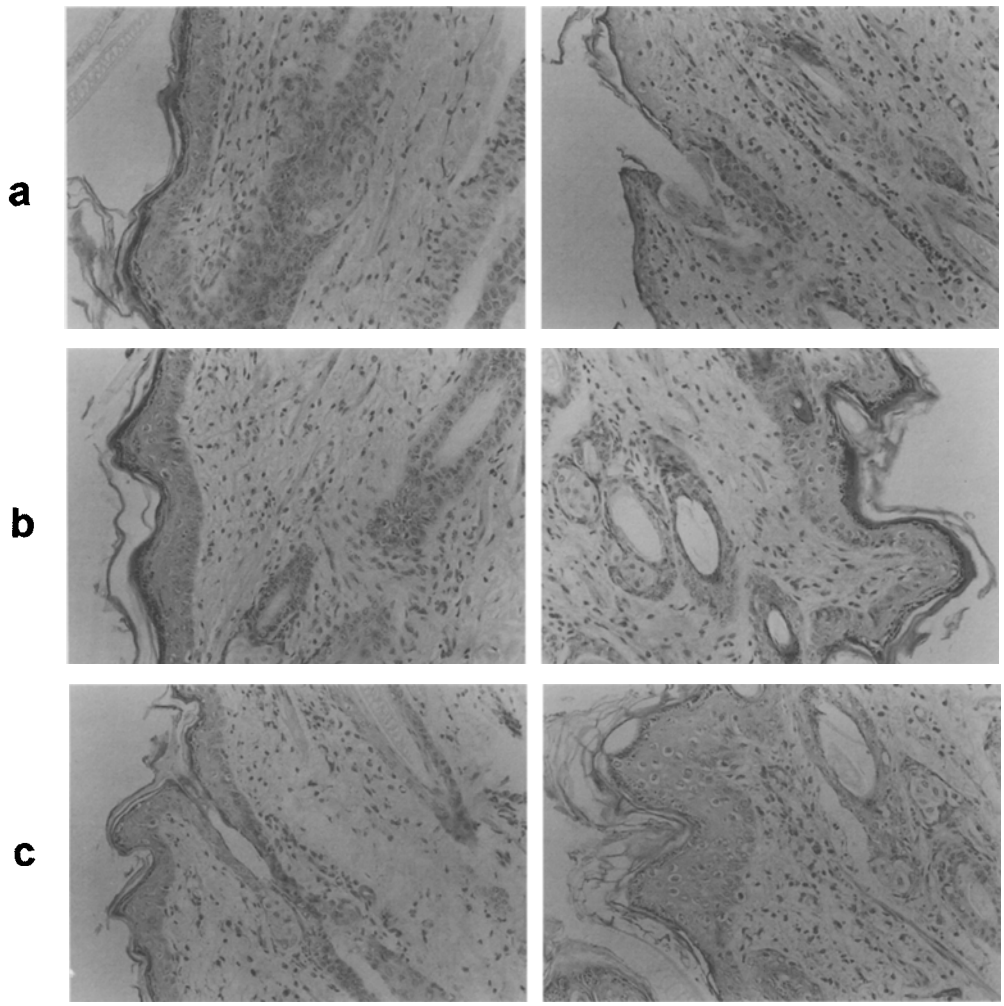


Fig. 7. Histopathological photomicrographs of skin tissue in guinea pigs. a: anthracene, b: sodium lauryl sulfate c: melatonin. Left: non-irradiation, right: irradiation.

ported from Lee *et al.* (1994) and Babu *et al.* (1992) reported which showed that MLT penetrated rapidly into human skin, tryptophan (precusor of MLT) produced siglet oxygen ($1O_2$) and superoxide radical (O_2^-), and these reactive forms of oxygen might contribute to membrane- and cytoplasm- damaging effects. The histopathological observation including degeneration in epidermis, vasodilation and severe hemorrhage in dermis induced by topical application of MLT could be recognized by the above reports. On the other hand, Poeggeler *et al.* (1994) reported that MLT was known to act as a potent endogenous free radical scavenger and Bangha *et al.* (1997, 1996) reported that UV-induced erythema was suppressed by topical treatment of MLT. But in this study, it is for me to predict that photoproducts of MLT absorb the UV radiation, and the photo-excited drug must produce reactive species either by chemical reaction or by transferring energy to other molecules, in particular to oxygen, to form an excited state of oxygen called siglet oxygen. The above prediction was supported by the findings reported by Kochevar (1989).

CONCLUSION

Photolysis of MLT was identified through the measurement of UV absorption spectra and ^{1}H-NMR spectra, however the structures of photoproducts were not identified. MLT was shown as positive in phototoxicity from the *in vitro* phototoxicity tests such as MTS assay but as negative in yeast growth inhibition assay. For the *in vivo* test using guinea pigs, very slight erythema was observed in the MLT (10, 1%)-treated groups 12 and 24 hr after irradiation. Histopathogenes such as degeneration, vasodilation and hemorrhage were observed in MLT (10%)-treated group 48 hr after irradiation. Consequently, MLT has proven to be a phototoxic material.

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