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Comparative Study on Red Blood Cell Hemolysis and Yeast Test by Photosensitizing Compounds

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광예민성화합물에 의한 적혈구 용혈현상과 Yeast 시험 비교연구

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ABSTRACT

In order to investigate the phototoxicity of five phenothiazine derivatives and one thioxanthen derivative were examined by using in vitro method based on growth inhibition of Candida albicans and red blood cell hemolysis. Effects of the test compounds on RBCs were monitored with a spectrophotometer and a drug PI in the Candida albicans was calculated on the basis of the lowest concentration giving a yeast-free zone. All phenothiazines phototoxic in the red blood cell hemolysis method were positive in the yeast test except promethazine. It was also observed that toxic photoproducts were formed by perphenazine and chlorpromazine in the red blood cell hemolysis.

INTRODUCTION

There is no in vitro laboratory test for assessing chemicals for their contact or photosensitizing powers if phototoxicity can be predetermined in vitro, the information will aid in prognosticating whether or not compounds have photoallergenic potential, because all phothoallergenic compounds are also phototoxic[1]. As the list of photosensitizing agents used in medicine and industry grows, demand for such a test increases. This report evaluates the phototoxic potential of chemicals by an in vitro test employing light to hemolyze red blood cells in the presence of photosensitizing compounds.

Irradiation (290~370 and 320~400 nm) of red blood cells in the presence of some photosensitizing compounds caused hemolysis. The extent of photohemolysis was determined to evaluate effects of these compounds on red blood cell membranes. Hemolysis of RBCs is a known photodynamic effects of some photosensitizing compounds. Clinically it becomes evident as purpura only in case of severe photodermatitis in which blood vessels are damaged[2,3].

The in vitro technique with Candida albicans was also found to be a sensitive method for demonstrating drug phototoxicity[4]. Thus we intended to compare the results of these two in vitro test. Chlorpromazine is capable of inducing phototoxic as well as photoallergic skin reations, as has been widely documented in clinical and experimental studies. Other phenothiazine derivatives also may enhance light sensitivity. Tested substances were five phonothiazines including chlorpromazine, perphenazine, trifluoperazine, thioridazine, promethazine, and thioxanthen derivative such as chlorprothixene. This report evaluates the phototoxic potential of chemicals by an in vitro test in the presence of photosensitizing compounds.

MATERIALS AND METHODS

1. Materials

Ultraviolet light of wave length 280~370 nm (emission peak 350 nm) was delivered by a Black light fluorescent tube 40 w. Sanyo Denki. Human red blood cells are convenient because of availability in most hospital laboratories. Blood stored up to 40 days been used in our experimental system without distorting the results. chlorpromazine (CPZ), perphenazine (PPZ), trifluoperazine (TFZ), thioridazine (TRZ), promethazine (PMZ), and chlorprothixene (CPX) used were U.S.P. These drug compounds were dissolved in appropriate solvents and the same amount of organic solvent was added to control solutions. Other chemicals used were of the highest quality commercially available.

2. Red blood cell hemolysis

Packed human RBCs were washed three times in physiological saline. Washed RBCs were added (0.2% v/v) to buffered drug solutions. The RBC-drug mixture was poured into Carrel tissue culture flasks. Control flasks were incubated in the dark at 37℃, while test flask were exposed to the Black light fluorescent tube for constant hours at 37℃ respectively (350 nm, output: 2.5 mW/cm at 10 cm). The control and exposed solutions were centrifuged for 10 min. at 3,000 rpm. Optical density of the supernatant fluid was read at 540 nm on a Beckman DU spectrophotometer. A 100% hemolysis solution was prepared by adding 0.02 m*l* of washed RBCs to 10 m*l* of a 0.04% NH_4OH solution. Results were expressed as percent relative to the 100% hemolyzed solution[5].

3. Evaluation of toxic photoproduct

5 m*l* of the drug compounds (50 μg/m*l*) in 0.05 M phosphate buffered saline (pH 7.4) were preexposed to the UVA for 30 min. and then added to RBCs solutions (0.2% v/v). After these mixtures were incubated for 30 min. at 37℃, and were centrifuged for 10 min at 3,000 rpm. Optical density of these supernatant fluid was read at 540 nm.

4. Alicans technique

The Candida screening test, a method by which phototoxicity was assayed in vitro, was

followed by the method of Daniels[4]. The phototoxic index (PI) for various drugs in the Candida albicans method was calculated by dividing the lowest concentration giving a yeast-free zone of 15 mm diameter by that of CPZ, which latter was given the 1.0.

RESULTS AND DISCUSSION

1. Red blood cell hemolysis

Drug-induced photosensitivity refers to adverse cutaneous responses which follow the combined or successive exposure to certain chemicals (photosensitizers) and to light[6]. Schulz *et al.*[7] studied the phototoxic activity of phenothiazines by using a variety of methods. Several phenothiazines have been reported to cause clinical phototoxicity[8,9].

Compared to using other cell systems to study photosensitization reactions, the human red blood cell has the advantage of being a single system essentially devoid of organelles, therfore RBC reactivity occurs primarily at the cell membrane. The molecular nature of the interaction remains unknown but the site of the reaction is at the membrane, probably with the protein of the lipoprotein shell[10].

Photohemolysis to five phenothiazines and one thioxanthen derivative was evaluated.

Table 1. Effect of doses of the drugs in phosphate buffered saline on red blood cell hemolysis by UVA* irradiation.

Drug	% Hemolysis treated with the drugs at the dose ($\mu g/ml$)				
	1	10	25	50	100
CPZ	0.40±0.38	3.50±0.46	43.24±0.59	85.50±0.91	96.08±2.00
PPZ	1.48±0.38	6.52±0.42	80.70±1.55	85.28±1.05	96.38±1.37
TFZ	1.22±0.46	8.84±0.40	58.54±0.81	92.38±1.40	97.28±1.07
TRZ	0.44±0.27	7.00±0.48	54.40±0.46	74.28±0.77	96.50±1.24
PMZ	0	0	0	0	0
CPX	0	0.24±0.23	5.08±0.43	4.74±0.75	5.08±0.93

Values are the mean±S.D. of 5 experiments.

*The Black light fluorescent tube for 10 min. at 37℃ (350 nm, 2.5 mW/cm)

Table 2. Effect of doses of the drugs in veronal buffered saline on red blood cell hemolysis by UVA* irradiation.

Drug	% Hemolysis treated with the drugs at the dose ($\mu g/ml$)				
	1	10	25	50	100
CPZ	2.22±0.35	7.08±0.70	82.34±1.30	93.58±1.13	97.26±0.88
PPZ	1.90±0.44	11.66±0.55	86.34±0.96	90.48±1.07	97.30±0.98
TFZ	2.16±0.56	13.20±0.60	90.58±0.71	97.24±0.84	98.20±0.71
TRZ	1.20±0.68	17.38±0.54	85.42±0.78	94.78±0.97	96.58±1.26
PMZ	0	0	0	0	0
CPX	0.10±0.14	0.54±0.31	5.96±0.63	6.20±0.73	6.52±0.56

Values are the mean±S.D. of 5 experiments.

*The Black light fluorescent tube for 10 min. at 37℃ (350 nm, 2.5 mW/cm)

Experiments to compare buffer solvent solutions with drugs were done at varying doses. We observed that many photosensitizers hemolyze RBCs in the presence of light sources (Table 1, 2). Most compounds photohemolyzed more in veronal buffer than in phosphate buffers. Photohemolysis may be a useful tool in screening drugs for phothsensitizing properties.

2. Evaluation of toxic photoproduct

Phototoxic reactions are broadly divided into those which are oxygen dependent and a lesser number which do not require oxygen. The mechanism of damage is one of colloid osmotic hemolysis mediated by peroxide formation following porphyrin excitation[6,11] and due to stable photoproducts since the drug irradiated alone failed to induce subsequent cell lysis. Our results (Table 3) showed that stable photoproducts were formed by perphenazine and chlorpromazine, but trifluoperazine and thioridazine were not formed photoproducts. So perphenazine and chlorpromazine produce oxygen-independent photohemolysis. On the other hand, the damage of trifluoperazine and thioridazine is one of colloid osmotic hemolysis mediated by peroxide formation following porphyrin excitation. In this study, irradation of chlorprothixene and promethazine had no effect on human serum complement activity.

Table 3. Comparison of preexposed drug with postexposed drug measured by photohemolysis.

Drug	% Hemolysis	
	preexposure*	postexposure**
CPZ	94.73±0.83	96.07±0.12
PPZ	97.80±1.41	94.13±0.15
TFZ	13.40±0.79	95.50±0.56
TRZ	2.67±0.72	94.14±0.76
PMZ	0	0
CPX	9.83±0.38	5.70±0.60

Values are the mean±S.D. of 5 experiments.

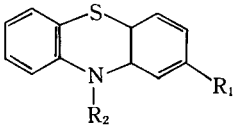
*drugs (50 $\mu g/ml$) were preexposed to UVA (350 nm, 2.5 mW/cm) for 30 min. and then 20 V/V% RBC (500 : 1) was added in phosphate buffered saline (pH 7.4, 0.05 M).

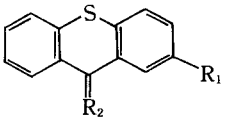
**exposure of the drug-RBC suspension to UVA (350 nm, 2.5 mW/cm) for 30 min. in phosphate buffered saline (pH 7.4, 0.05 M).

3. Albicans technique

The capacity of defferent drugs to inhibit the out-growth of Candida albicans exposed to UVA in vitro culture was studied. The Candida screening test of Daniels, a method by which phototoxicity was assayed in vitro, failed with some well known photosensitizers[12,13]. But albicans technique was also found to be a sensitive method for demonstrating phototoxicity. In our study, all phenothiazines phototoxic in the red blood cell hemolysis method were positive in the yeast test except PMZ (Table 4). In the mouse tail method, an in vivo technique based on the inflammatory response of the mouse tail after systemic administration of the drug plus UVA irradiation, CPZ was found more potent than PPZ, TFZ and TRZ. PMZ had also activity as like yeast test. On the other hand, CPZ, lacking nitrogen in this structure, showed no activity in this vivo method. With the red blood cell hemolysis and candida albicans method, CPZ, PPZ, TFZ and TRZ showed a phototoxic potency. CPX was also found poor activity in this two method and no activity in the mouse tail method. PMZ had no activity in the red blood cell hemolysis but showed activity in the yeast test and mouse tail method above. Therefore, the results of this two method were in aggrement with each other in many case but not absolutely. Consequently, the yeast method and photohemolysis appears to be useful as a

Table 4. Phototoxic index (PI) in vitro yeast test for drugs.

phenothiazine deriv. 

thioxanthenes.* 

Drug	R_1	R_2	PI
CPZ	Cl	$CH_2CH_2CH_2N(CH_3)_2$	1.0
PPZ	Cl	$CH_2CH_2CH_2N$◯NCH_2CH_2OH	0.9±0.1
TFZ	CF_3	$CH_2CH_2CH_2N$◯NCH_3	1.9±0.3
TRZ	SCH_3	CH_2CH_2◯ N CH_3	0.6±0.1
PMZ	—	$CH_2CHN(CH_3)_2$ CH_3	4.6±0.5
CPX*	Cl	$=CHCH_2CH_2N(CH_3)_2$	27.4±1.7

The phototoxic index (PI) for various drugs was calculated by dividing the lowest concentration giving a yeast-free zone of 15 mm diameter by that of CPZ, which latter was given the 1.0. Values are the mean±S.D. of 5 results.

screen test but the results should be checked with an in vivo method.

REFERENCES

1. Willis, I. and Kligman, A.M.; The mechanism of photoallergic contact dermatitis, *J. Invest. Derm.*, **51,** 378 (1968)
2. Cook, J.S.; Some characteristics of hemolysis by ultraviolet light, *J. Cell Comp. Physiol.*, **47,** 55 (1956)
3. Blum, H.F.; Photodynamic action and disease caused by light, Reinhold Publishing Co., New York, 185 (1941)
4. Daniels, F.; A simple microbiological method for demonstrating phototoxic compounds, *J. Invest. Derm.*, **44,** 259 (1965)
5. Kahn, G. and Fleischaker, B.; Red blood cell hemolysis by photosensitizing compounds, **56,** 85 (1971)
6. Harber, L.C. and Baer, R.C.; Pathogenic mechanisms of drug induced photosensitivity, *J. Invest. Derm.*, **58,** 327 (1972)
7. Schulz, K.H., Wiskemann, A. and Wulf, K.; Klinische und experimentell untersuchungen uber die photodynamisch wirksamkeit von phenotheazinderivaten, insbesondere von megaphen, *Arch Dermatol. Forsch.*, **202,** 285 (1956)
8. Ippen, H.; Lichtbeeinflusste arzneimittel neben wirkungen an der haut, Dtsch. Med. Wochenschr, **87,** 480 (1962)
9. Pathak, M.A. and Epstein, J.H.; Normal and abnormal reactions of man to light, In Dermatology in General Medicine, McGraw-Hill, New York, 412 (1971)
10. Cook J.S. and Blum, H.F.; Dose relationships and oxygen dependence in ultraviolet and photodynamic hemolysis, *J. Cell Comp. Physiol.*, **53,** 41 (1956)
11. Emmett, E.A.; Toxic responses of the skin, Macmillan, New York, 412 (1986)
12. Ljunggren, B. and Moller, H.; Phenothazine phototoxicity: an experimental study on chlorpromazine and related tricyclic drugs, Act. Derm. (Stockholm), **57,** 325 (1977)

13. Webster, G.F., Kaidbey, K.H., and Kligman, A. M.; Phototoxicity from benoxaprofen: in vivo and in vitro studies, Photochem. Photobiol., **36,** 59 (1982)