Acta Dermatovener (Stockholm) 51: 345–349, 1971

A GRAVIMETRIC TECHNIQUE FOR THE ANALYSIS OF ACUTE ULTRAVIOLET DAMAGE

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Abstract. The authors have developed a simple laboratory model with a blacklight source for the gravimetric analysis of acute ultraviolet damage. The use of weight in this bioassay method is precise and simple which is in contrast to the use of color as a measure of UV damage.

Evaluation of sunscreen preparations has consisted primarily of the subjective measurement of erythema in humans and animals after the application of topical protective agents and subsequent exposure to natural sunlight or artificial light sources. Kooyers (10), in measuring the sunlight protective effect of dihydroxyacetone (DHA)/naphthoquinone, described a method of evaluation which involved photosensitizing white rats, exposing their paws to sunlight, and measuring the increase in weight of the "sunburned" edematous paws. Thus, a gravimetric measure of acute ultraviolet damage, *edema,* was used instead of the subjective visual estimation of the erythematous response.

Using the Kooyer's technique, we were unable to reproduce consistently the control parameters of the method; therefore, we have developed a laboratory model with a blacklight light source for the gravimetric analysis of acute ultraviolet radiation damage.

METHODS

Adult male rats (Holtzman random breed albino) were given orally 30 mg/kg 8-methoxypsoralen in glyceryl tributyrate (tributyrin) 90 min prior to light exposure. Sixty minutes later, they received 40 mg/kg sodium pentobarbital (Nembutal) by intraperitoneal injection. Since rats are not normally photosensitive to blacklight, we photosensitized them with the oral administration of 8-methoxypsoralen which causes photosensitization to a narrow band in the ultraviolet (UV) spectrum with a peak absorption at 360 nm (12, 4). The unexposed hind paw (standard weight control) in each animal was masked with a finger cloth from a canvas work glove (Wards, PowrHouse No. 42-351). The rats were then placed in 64 mm diameter pyrex tubes horizontally in a rack over the light source consisting of two GE Blacklight fluorescent tubes (GE F15T8 Blacklight fluorescent tubes have an emission spectrum from 320 to 400 nm with a peak from 360 to 380 nm) in a standard X-ray view box from which the diffusing glass had been removed (Figs. 1 and 2). The pyrex tubes were 10.5 cm above the blacklight tubes (top of fluorescent tube to the bottom of pyrex tube) and at right angle to the longitudinal axis of the blacklight tube.

At the desired time after light exposure, the rats were sacrificed with either intraperitoneal injection of sodium pentobarbital or exposure to ether. The hind paws were amputated at the tibia-fibula/talar joint using a scalpel with a No. 11 surgical blade and weighed on an analytical balance to the nearest milligram (Mettler Model H5).

In evaluating Kooyers' technique, we used his animal restrainers. The stock consisted of a flat board which was wide enough to hold rats at right angle to the long axis of the board. Each animal had its own restraining position. Two holes in the board beneath each animal allowed the paws to be pulled through and exposed to light. The animals used in the evaluation of his method were exposed to 2 hours of blacklight and sacrificed at 72 hours.

The following experiments were designed to define the control parameters of our animal model: (1) minimal exposure time to produce UV damage (edema), (2) optimum time for sacrifice of animals, and (3) length of time animal retains psoralens and remains photosensitized. In order to determine the least time necessary to produce minimal amount of edema, the animals were exposed for $^1/_4$, $^1/_2$, 1, and 2 hours and sacrificed at 72 hours. The optimum time for edema reaction was determined by exposing the animals to blacklight (2 hours) and sacrificing them at 24, 48, and 72 hours. The final parameter, the length of time the animals remain photosensitized from one dose of psoralen, was determined by giving one hour of UV exposure at the end of the following periods after the oral psoralen dose: $1\,^1/_2$, $2\,^1/_2$, $3\,^1/_2$, and $4\,^1/_2$ hours. The animals were sacrificed 48 hours after the initial exposure in this latter experiment.

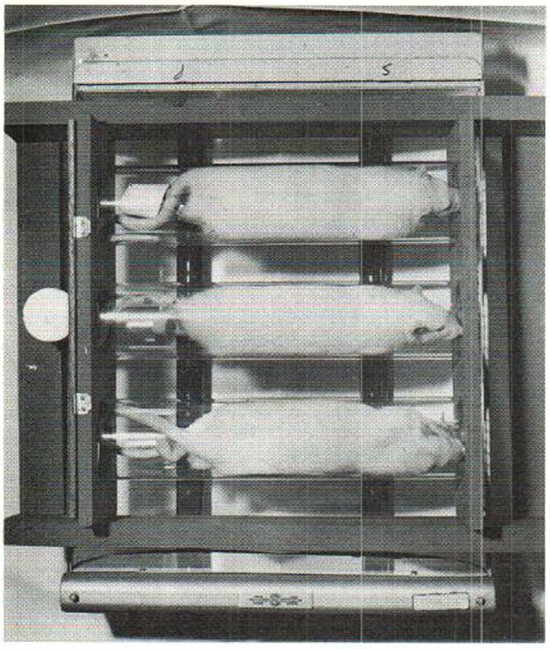

Fig. 1. Top view of the apparatus.

The results are reported as the percentage difference of the weights of the control and exposed hind paws and the difference of the gram weight of the paws.

RESULTS

Table I shows the results of the comparison of the weight of the normal, unexposed left and right amputated hind paws. The data indicate the weights are remarkably constant despite the free-hand amputation of the paws at the tibia-fibula/talar joint. However, there is a significant difference in the weight of the amputated paws. The left paw appears to be favored in the cutting process and shows a definite bias in its average increased weight. That small bias, however, is not of any consequence when one observes the data in the subsequent tables. Table II reveals that the Kooyers' method had some major difficulties in techniques as the variability of the data was larger

Table I. *The consistency of the weight (g) of the hind paws (controls)*

Right	Left	Diff.	%
1.81	1.82	−0.01	0.5
1.73	1.75	−0.02	1.1
1.79	1.74	+0.05	2.9
1.75	1.75	0.00	0.0
1.70	1.73	−0.03	1.7
1.82	1.85	−0.03	1.6
2.02	2.08	−0.06	2.9
1.93	1.90	+0.03	1.6
1.75	1.78	−0.03	1.7
2.10	2.16	−0.06	2.8
1.95	1.97	−0.02	1.0
1.95	1.98	−0.03	1.5
1.99	2.01	−0.02	1.0
1.85	1.90	−0.05	2.6
2.22	2.26	−0.04	1.8
2.76	2.78	−0.02	0.7
1.86	1.88	−0.02	1.1
1.54	1.51	+0.03	2.0
1.64	1.65	−0.01	0.6
2.25	2.22	+0.03	1.4
Mean		−0.015	1.5
S.D.		0.030	0.8
S.E.		0.007	0.2

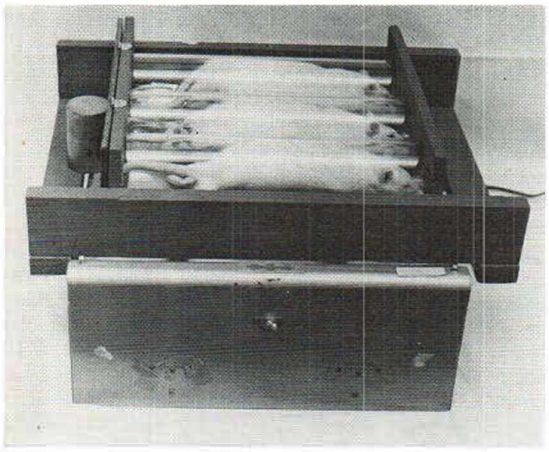

Fig. 2. Side view of the apparatus.

Table II. *Weight difference after UV exposure of the right paw by the Kooyer's technique*[a]

Exposure time (min)	Right	Left	Diff.	%
120	2.34	2.26	+0.08	3.5
120	2.52	2.05	+0.47	22.9
120	2.33	2.30	+0.03	1.3
120	2.30	2.23	+0.07	3.1
120	2.80	2.47	+0.33	13.4
120	2.70	2.38	+0.32	13.4
120	2.79	2.14	+0.65	30.4
120	2.26	2.08	+0.18	8.7
Mean			0.266	12.1
S.D.			0.218	10.3
S.E.			0.077	3.6

[a] Animals sacrificed at 72 hours after UV exposure.

than our method (compare Table II with Table III, 120 min). Note the difference in the means and their standard deviations (S.D.). We observed that during the light exposure with the Kooyers' technique, the animals needed constant attention (in direct contrast to our method) to keep the rats in position so that they did not retract their hind paws through the holes, thereby reducing the UV exposure. Table III indicates that the minimal amount of UV exposure necessary to produce significant increase in edema of the paws (weight) was $^1/_2$ hour. Only 15-min intervals were measured. Shorter intervals of 5 min may define this lower limit more precisely; but for the practical purposes of our future use, 15 min was considered adequate. There was no overlap of values between the 15 min and 30 min exposures. Table IV demonstrates that the optimum sacrifice time for the rats was either 48 or 72 hours. The data in Table V shows that the rats remained photosensitive throughout 4 hours of UV exposure, thus confirming previous findings (1, 5).

Table III. *Quantitation of UV exposure necessary to produce minimal weight change*[a]

Min	Right	Left	Diff.	%
15	2.06	2.05	+0.01	0.5
15	1.95	1.95	0.00	0
15	1.99	2.01	−0.02	1.0
Mean			−0.003	0.5
S.D.			0.015	0.5
S.E.			0.009	0.3
30	2.27	1.94	+0.33	17.0
30	2.78	1.99	+0.79	39.7
30	2.21	2.02	+0.19	9.4
30	2.20	2.04	+0.16	7.8
Mean			0.368	18.5
S.D.			0.291	14.7
S.E.			0.146	7.4
60	3.04	2.06	+0.98	47.6
60	2.74	2.02	+0.72	35.6
60	2.64	2.23	+0.41	18.4
60	2.53	1.95	+0.58	29.7
Mean			0.673	32.8
S.D.			0.241	12.2
S.E.			0.120	6.1
120	2.57	2.02	+0.55	27.2
120	2.73	2.13	+0.60	28.2
120	2.77	2.23	+0.54	24.2
120	2.75	2.01	+0.74	36.8
120	2.63	2.13	+0.50	23.5
120	2.78	2.07	+0.71	34.3
120	2.88	2.09	+0.79	37.8
120	2.67	2.08	+0.59	28.4
Mean			0.628	30.1
S.D.			0.106	5.5
S.E.			0.037	2.0

[a] Animals sacrificed at 72 hours after UV exposure.

Table IV. *Time of sacrifice after UV exposure*

Two hours exposure

Hours	Right	Left	Diff.	%
24	1.74	1.67	+0.07	4.2
24	2.08	2.04	+0.04	2.0
Mean			0.055	3.1
S.D.			0.021	1.6
S.E.			0.015	1.1
48	2.64	1.89	+0.75	39.7
48	3.47	2.37	+1.10	46.4
48	2.70	2.10	+0.60	28.6
48	2.61	1.94	+0.67	34.5
48	3.22	2.19	+1.03	47.0
48	2.75	2.19	+0.56	25.6
48	3.33	2.45	+0.88	35.9
Mean			0.798	36.8
S.D.			0.211	8.2
S.E.			0.080	3.1
72	See Table III, 120 minutes			

DISCUSSION

Erythema has long been used as an endpoint in most studies of UV protection. The use of color has many difficulties (2, 3). The most obvious disadvantage is that it is subjective and varies with the color perception of the observer whose individual variability may be great from one observation to the next. The variability of color perception becomes even greater from one investigator to another. Many individuals have subtle visual incapacities for color perception (color blindness). Besides the difficult decision of what is red, one must define the term a perceptible erythema. Does one mean the faintest observation of erythema seen or an erythema which fills all of the exposed site? In addition, investigators use different time periods after irradiation to observe color change (i.e., 8, 12, 24 and 48 hours, etc.). Furthermore, the cutaneous vascular tree has a circadian rhythm which significantly influences the erythematous response of the skin (8, 9). In short, the criterion of erythema evaluation is very variable and difficult in comparative studies.

Previously in any investigative program with

Table V. *Photosensitivity period of the rats*

UV exposure 1 hour, sacrificed at 48 hours. Figures in italics designate the paw which was exposed to UV light

Hours[a]	Right	Left	Diff.	%
1½	**2.91**	2.25	+0.66	29.3
1½	**3.05**	2.31	+0.74	32.0
Mean			0.7	30.7
S.D.			0.057	1.9
S.E.			0.040	1.4
2½	2.03	**2.33**	+0.30	12.9
2½	**3.02**	2.22	+0.80	36.0
2½	1.97	**2.62**	+0.65	24.8
2½	**2.18**	1.99	+0.19	9.5
Mean			0.485	20.8
S.D.			0.287	12.1
S.E.			0.144	6.0
3½	1.95	**2.31**	+0.36	15.6
3½	**2.51**	1.98	+0.53	26.8
3½	2.16	**2.56**	+0.40	15.6
3½	**2.35**	1.98	+0.37	18.7
Mean			0.415	19.2
S.D.			0.079	5.3
S.E.			0.039	2.6
4½	2.18	**2.66**	+0.48	18.0
4½	**2.09**	1.80	+0.29	16.1
4½	2.37	**2.70**	+0.33	12.2
4½	**2.59**	2.25	+0.34	15.1
Mean			0.36	15.4
S.D.			0.083	2.4
S.E.			0.041	1.2

[a] That period of time from the oral dose of psoralen to the onset of UV exposure.

sunscreens, the use of human subjects was the only practical technique. There are many inherent difficulties when using volunteers. There is marked variability in erythemal response which is dependent on such factors as 1) cutaneous location of test sites, 2) test subject's thickness of epidermis and stratum corneum, 3) racial and individual pigmentary differences (11), and (4) the designated time of the day for the measurement of the erythema (circadian rhythm) (8, 9). In addition, there are the difficulties in obtaining human volunteers with or without incentive pay and meeting governmental agencies' requirements for the use of human subjects in clinical studies. In any preliminary or developmental testing program, the latter obstacles may be financially prohibitive. With our animal model, the use of human test subjects can be postponed until the final clinical testing phase.

The use of natural sunlight by Kooyers limits the test procedure he reported. It also creates difficulty in the standardization of the light exposure because of the factors which influence sunlight intensity: 1) time of day, 2) time of year, 3) sky cover (clouds), and 4) local atmospheric conditions (pollution). These factors make any comparison study difficult between two investigations from different climatic areas.

By using the animal laboratory model, many of the above-mentioned variables are eliminated. In addition, the rats are closely matched with respect to age and genetic background. The problem of variability of sunlight energy is eliminated by the use of the blacklight. With our model, constant test conditions are available throughout the year. The apparatus is inexpensive and the measurement of weight is simple and highly reliable.

In order to eliminate the problem of determining the energy output of the blacklight, we have defined a unit of time exposure with the apparatus designed at Minnesota. This standard can be defined in terms of the biological response of the animals and therefore not directly dependent upon a physical measurement of the energy output of the fluorescent tubes. The minimal exposure time necessary to cause an edematous response is the unit measurement and exposure times can be recorded as multiples of the unit rather than minutes or hours; therefore, we propose that the unit be designated a Minnesota Minimal Paw Unit for the specific biologic and physical conditions described in this report.

We developed the animal model to test the DHA/lawsone concept of sunlight protection. We have previously reported that the treatment of stratum corneum with DHA/lawsone decreased the transmission of UV through the keratin not only in the far UV but also in the near UV (13, 6). Thus, in measuring the protective effect of DHA/lawsone, an evaluation of the protective effect in the near UV is presumptive evidence of the protective effect in the far UV. In a preliminary experiment (7), we have verified the effectiveness of DHA/lawsone. Twenty-four hours prior to blacklight exposure, one hind paw of each rat was dipped six different times (at least 1 hour apart) into freshly prepared solution of 3% DHA and 0.13% lawsone in 50% isopropyl alcohol/distilled water. The solution was prepared before each application by mixing equal parts of the two

following stable stock solutions: (*a*) 6% DHA in 50% isopropanol/distilled water, and (*b*) 0.25% lawsone in 50% isopropanol/distilled water. The next day the animals were photosensitized as above. The untreated hind paws were masked. The animals were exposed to blacklight and sacrificed at 48 hours. The animals were exposed up to 4 hours of UV. The average weight gain in the 4-hour group was 7% difference; therefore, the animals received significant protection. This confirms Kooyers' (10) observation.

Sunburn fluorescent tubes (less than 320 nm emission) can be substituted for the blacklight tubes and the rats need not be given psoralens to photosensitize them. The sunburn tubes would have to be used in any study of sunscreens which protect only in the far UV; however, for our study of the DHA/lawsone sunscreen, the use of the near UV is adequate. If the sunburn tubes are used, the pyrex tubes must be replaced by quartz or wire mesh restraining tubes. A decided advantage of the blacklight tubes is that their radiation is not dangerous to the laboratory worker. This can be very important as we envision our animal model will have possible use as an assay method for anti-inflammatory agents, such as steroids.

ACKNOWLEDGEMENTS

We wish to thank W. Kooyers for furnishing us with the animal stocks used in his original experiments and Dr W. L. Fowlks for supplying us with psoralens and advice.

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Received February 15, 1971

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