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TECHNICAL REPORT

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The Repair of CPDs in Cell Culture Over 24 Hours

Using Antibodies Against CPD

August 23, 2004

Summary

Human HaCaT keratinocyte cells were treated with AC-11 and irradiated with UVB. DNA damage persisting at 24 h was assayed by immunofluorescence. Cells incubated before and after with AC-11 showed 16-25% fewer CPDs ($p<0.001$) than untreated cells. Coloring of the nuclei of cells treated with AC-11 were noted at 24 h.

Introduction

Solar UV creates DNA damage in skin by linking adjacent bases to form cyclobutane pyrimidine dimers (CPD). These may be of the thymine-thymine type, or they may be between adjacent cytosines, or other combinations. CPDs are slowly removed from the DNA by a natural excision repair process, which removes about 50% of the CPDs in 24 h. The purpose of the study was to determine the effect of AC-11 on repair of CPDs in keratinocyte cell culture over 24 h.

Methods

Test article: AC-11 (C-MED-100), powder, lot #0229052006

This study measured the repair of CPDs in cell culture over 24 h using antibodies against CPD. HaCaT (keratinocyte line) cells were seeded on glass slides, and pretreated for 24 h with 3 concentrations of the test article. Two samples were untreated. The cells were irradiated with 500 J/m^2 UV-B (Kodacel filtered FS40 sunlamp). One vehicle treated sample was fixed immediately for assay. The remaining samples were incubated with the appropriate test article for 24 hrs. At that point, all samples were fixed and stained with antibodies specific for CPD in DNA. The binding was visualized by fluorescent

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secondary antibody and fluorescence microscopy. CPDs were measured by a semi-quantitative scoring system. Statistical analysis was by ANOVA (Instat, GraphPad).

Results

The cells appeared attached to the slides throughout the experiments. Some rounding up of the cells occurred in the treated samples at 24 h. Cells treated with AC-11 concentrations of 1.5% and 3% for 24 h had a brown color concentrated in the nuclei visible under 60X phase-contrast microscopy. The effect of this coloring on the fluorescence assay is unknown. The results of the CPD scoring are shown in Table 1.

Table 1. CPD scoring results

UV	Treatment	Incubation Time (h)	CPD score	Standard Deviation	Number of Observations
None	None	0	0.58	0.50	50
500 J/m^2	None	0	3.38	0.61	100
500 J/m^2	None	24	3.55	0.64	100
500 J/m^2	AC-11, 0.5% (5 mg/ml)	0	2.84	0.68	50
500 J/m^2	AC-11, 0.5% (5 mg/ml)	24	2.81	0.92	100
500 J/m^2	AC-11, 1.5% (15 mg/ml)	0	3.76	0.43	50
500 J/m^2	AC-11, 1.5% (15 mg/ml)	24	2.66	0.82	50
500 J/m^2	AC-11, 3.0% (30 mg/ml)	0	3.08	0.81	100
500 J/m^2	AC-11, 3.0% (30 mg/ml)	24	2.98	0.60	100

The data of interest was the CPD score at 24 hr among the untreated and treated samples. These results are shown graphically in Figure 1.

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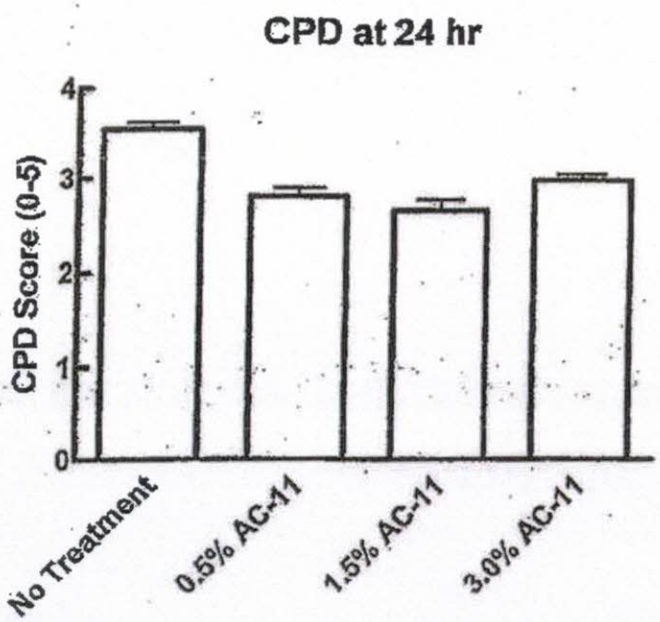
Figure 1. CPD Score in NHEK at 24 hr after UVB irradiation. Treatment with AC-11 as indicated. Error bars are standard error of the mean.



The percent difference was calculated as the difference in CPD score between "no treatment" and treatment scores, divided by the "no treatment" score. The raw data were analyzed for statistical significance using the Kruskal-Wallis Test for nonparametric ANOVA. The nonparametric method was used because the parametric ANOVA assumes equivalent standard deviations and the Bartlett test (p=0.015) suggested that the differences among the standard deviations were significant. The results of the ANOVA are shown in Table 2. The statistical analysis is appended

Comparison	Percent Difference	p-value
UV-24h v 0.5% AC-11	21%	p<0.001
UV-24h v 1.5% AC-11	25%	p<0.001
UV-24h v 3.0% AC-11	16%	p<0.001

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Appendix

AC-11 CPD at 24 hr

Col. title	No UV 0h	UV 0h	UV 24h	0.5% 0h
Mean	0.58	3.38	3.55	2.84
Standard deviation (SD)	0.4986	0.6159	0.6416	0.6809
Sample size (N)	50	100	100	50
Std. error of mean (SEM)	0.07051	0.06159	0.06416	0.09630
Lower 95% conf. limit	0.4382	3.258	3.423	2.646
Upper 95% conf. limit	0.7218	3.502	3.677	3.034
Minimum	0.000	2.000	2.000	2.000
Median (50th percentile	1.000	3.000	4.000	3.000
Maximum	1.000	4.000	4.000	4.000
Normality test KS	0.3802	0.3561	0.3802	0.2729
Normality test P value	<0.0001	<0.0001	<0.0001	0.0012
Passed normality test?	No	No	No	No

Col. title	0.5% 24h	1.5% 0h	1.5% 24h	3% 0h
Mean	2.81	3.76	2.66	3.08
Standard deviation (SD)	0.9178	0.4314	0.8234	0.8125
Sample size (N)	100	50	50	100
Std. error of mean (SEM)	0.09178	0.06101	0.1164	0.08125
Lower 95% conf. limit	2.628	3.637	2.426	2.919
Upper 95% conf. limit	2.992	3.883	2.894	3.241
Minimum	1.000	3.000	1.000	1.000
Median (50th percentile	3.000	4.000	3.000	3.000
Maximum	4.000	4.000	4.000	4.000
Normality test KS	0.2686	0.4710	0.2686	0.2708
Normality test P value	0.0015	<0.0001	0.0015	<0.0001
Passed normality test?	No	No	No	No

Col. title	3% 24h
Mean	2.98
Standard deviation (SD)	0.6027
Sample size (N)	100
Std. error of mean (SEM)	0.06027
Lower 95% conf. limit	2.860
Upper 95% conf. limit	3.100
Minimum	1.000
Median (50th percentile	3.000
Maximum	4.000
Normality test KS	0.3432
Normality test P value	<0.0001
Passed normality test?	No

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AC-11 CPD at 24 hr

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is < 0.0001, considered extremely significant.
Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
UV 24h	100	23566	235.66
0.5% 24h	100	15076	150.76
1.5% 24h	50	6477.0	129.54
3% 24h	100	16307	163.07

Kruskal-Wallis Statistic KW = 60.626 (corrected for ties)

Dunn's Multiple Comparisons Test

Comparison	Mean Rank Difference	P value
UV 24h vs. 0.5% 24h	84.905	*** P<0.001
UV 24h vs. 1.5% 24h	106.12	*** P<0.001
UV 24h vs. 3% 24h	72.595	*** P<0.001

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
UV 24h	100	4.000	2.000	4.000
0.5% 24h	100	3.000	1.000	4.000
1.5% 24h	50	3.000	1.000	4.000
3% 24h	100	3.000	1.000	4.000

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