Establishment and use of 3t3 NRU assay for assessment of phototoxic hazard of cosmetic products

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Abstract

Objective: To establish an alternative 3T3 mouse fibroblast neutral red uptake assay and investigate the feasibility of utilizing an alternative in vitro method to replace the animal testing, in particular, in phototoxicity assessment of cosmetics.

Method: Fifteen phototoxic and nine non-phototoxic chemicals were tested and 20 functional cosmetic products were assessed. The mean photo effect (MPE) and the photo-irritation factor (PIF) of Balb/c3T3 cells were analyzed after exposure to specific chemicals and ultraviolet radiation, and then compared with the results of whole-animal phototoxicity testing.

Result: A negative result was obtained with all the non-phototoxic chemicals in the in vitro test, while fourteen out of the fifteen phototoxic chemicals gave a positive result. The Pearson's chi-square test indicated no statistically significant difference from the historic phototoxicity data. For the assessed 20 cosmetic products, a high correlation was found between in vitro and in vivo data.

Conclusion: The alternative 3T3 NRU phototoxicity assay was established successfully as a substitute for the guinea pig-based phototoxicity test in assessing the phototoxic effects of cosmetics.

Keywords: phototoxicity test, alternative test, in vitro test, 3T3 fibroblast, neutral red

Along with the rapid economic growth and social development, the cosmetics market witnesses an unprecedented expansion in China. In particular, the turnover of functional cosmetics is increasing at a rate above 10%. However, the broad variety of constituents of personal care products could present a potential phototoxic hazard. Permissions by the regulatory authority are required for manufacture and marketing of functional cosmetics in order to ensure the cosmetics are safe to use. Widely used for phototoxicity assessment of cosmetics, animal testing is controversial due to moral issues. In recent years, it has become a global trend to introduce in vitro alternative methods into phototoxicity evaluation [1]. In vitro 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) has been approved and recommended by European Union and OECD to be used for phototoxicity assessment of cosmetic products [2-5]. China has started research and utilization of alternative methods to replace animal testing lately and the present study is intended to establish an in vitro 3T3 NRU PT assay with fibroblast cells and to investigate the validity of this method and the feasibility of replacing animal testing in assessing the phototoxic effects of cosmetics.

1. Materials and methods

1.1 Test strain

3T3 fibroblasts Source: Institute of Tumor, Zhongshan Medical University

1.2 Test substances

The test chemicals were selected in accordance with the criteria adopted by the working group of European Center for the Validation of Alternative Methods ("EVCAM"), including 16 phototoxic substances and 8 non-phototoxic substances, all purchased from Sigma. Other chemical reagents used in the tests were of analytical or higher purity. Cosmetic products under evaluation were test samples registered with Center for Disease Control and Prevention of Guangdong Province ("CDCP") during the 2004-2005 period, 20 varieties in total, for
sun protection, spot removing and hair nourishing purposes respectively.

1.3 Laboratory animal

Adult albino guinea pigs (250g-300g) were supplied by Laboratory Animal Center, Southern Medical University. Animal health certificate: No. 0000831. 6 animals were used for each test sample. There was no limitation on whether female or male animal should be used. Animals were subject to 3-5 days of quarantine and adaptation before test. Animal use permit: No. SYXK (YUE) 2003-0011.

1.4 Test methods

1.4.1 3T3 NRU assay

3T3 cells were cultured in 96-well plates for twenty-four hours before treated with chemicals of eight concentrations for one hour and exposed to UVA (1.67mW/cm). A parallel culture was not irradiated. The chemicals were washed away after fifty minutes. The cells were incubated in the culture medium containing neutral red for three hours and then washed. The optical density of NRU (540nm) was measured with a microplate reader.

1.4.2 Whole-animal test

In compliance with Hygienic Standard for Cosmetics (2002).

1.5 Statistics and data analysis

Given the measured optical density, prediction models for mean photo effect ("MPE") and photo irritation factor ("PIF") was established respectively by use of PHOT032. Test results were analyzed and an analysis of correlation between in intro and in vivo data was made.

2. Results

2.1 Repeated tests of PIF-based classification

The test procedure was repeated five times for 3 phototoxic substances and 2 non-phototoxic substances. The results obtained with PIF-based classification were highly reproducible (Table 1).

2.2 Efficacy analysis of PIF value based classification

PIF values were used for classification of phototoxicity potential. 14 phototoxic substances and 9 non-phototoxic substances were identified correctly. Only one phototoxic substance (amiodarone) was falsely classified as non-phototoxic. A Pearson chi-square test showed that the results of 3T3 NRU assay were highly correlative with historic in vivo data (Table 2).

2.3 Repeated tests of MPE-based classification

The test procedure was repeated five times for 3 phototoxic substances and 2 non-phototoxic substances. The results obtained with MPE-based classification were highly reproducible (Table 3).

2.4 Efficacy analysis of MPE value based classification

MPE values were used for classification of phototoxicity potential. 14 phototoxic substances and 9 non-phototoxic substances were identified correctly. Only one phototoxic substance (amiodarone) was falsely classified as non-phototoxic. A Pearson chi-square test showed that the results of 3T3 NRU assay were highly correlative with historic in vivo data (Table 4).

2.5 Results of 3T3 NRU assay of 20 cosmetic products

PIF and MPE values obtained in the 3T3 NRU assay of 20 cosmetic products indicated no phototoxic potential.

2.6 Results of animal-based phototoxicity test of 20 cosmetic products

Results of animal-based phototoxicity test of 20 cosmetic products indicated no phototoxic potential.

2.7 Correlation between results of 3T3 NRU assay and in vivo phototoxicity test

The comparison of 3T3 NRU/animal-based classification of phototoxic potential indicated no significant difference (Table 5).

3. Discussion

The prediction models for MPE and PIF: [6] [7] respectively were established through the present study by analyzing the concentration response curves.
Table 3. Repeated tests of MPE-based classification

<table>
<thead>
<tr>
<th>Substance</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; time</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; time</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; time</th>
<th>4&lt;sup&gt;th&lt;/sup&gt; time</th>
<th>5&lt;sup&gt;th&lt;/sup&gt; time</th>
<th>CV</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-methyl coumarin</td>
<td>0.378</td>
<td>0.442</td>
<td>0.35</td>
<td>0.326</td>
<td>0.37</td>
<td>0.116</td>
<td>+</td>
</tr>
<tr>
<td>Methoxsalen</td>
<td>0.521</td>
<td>0.557</td>
<td>0.535</td>
<td>0.592</td>
<td>0.614</td>
<td>0.069</td>
<td>+</td>
</tr>
<tr>
<td>Anthracene</td>
<td>0.731</td>
<td>0.694</td>
<td>0.625</td>
<td>0.599</td>
<td>0.688</td>
<td>0.081</td>
<td>+</td>
</tr>
<tr>
<td>Octyl Salicylate</td>
<td>0.018</td>
<td>0.016</td>
<td>0.003</td>
<td>0.019</td>
<td>0.018</td>
<td>0.452</td>
<td>-</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.002</td>
<td>0.042</td>
<td>0.014</td>
<td>0.056</td>
<td>0.043</td>
<td>0.716</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4. Efficacy analysis of MPE value based classification for 24 substances

<table>
<thead>
<tr>
<th>Animal test</th>
<th>3T3 test</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>10</td>
</tr>
</tbody>
</table>

Note: The result of Pearson's chi-square (p>0.05) indicated no statistically significant difference between the results of the two test methods.

Table 5. Comparison of results of PIF/MPE based classification with results of animal test for 20 cosmetic products

<table>
<thead>
<tr>
<th>Animal test</th>
<th>3T3 test</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

Reference:


10. Spielmann H, Balls M, Dupuis J, et al. The international EU/COLIPA in vitro phototoxicity validation study results of Phase II (blind trial); part 1: the 3T3 NRU phototoxicity test. TIV; 1998(12): 305-327.


