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In vitro Alternatives to Skin Irritation Test

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Abstract—*In vitro* cell culture system has been proposed as a promising alternative model to *in vivo* skin irritation test. These studies were performed to screen the cytotoxicity effects of surfactants using normal human skin fibroblasts. Cell membrane integrity assessed by the leakage of lactate dehydrogenase (LDH) and mitochondrial integrity by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction test were affected in a dose dependent manner. The irritation potential of surfactants to human skin patch test, and the changes of capillary permeability by rabbit intradermal safety test were assessed as *in vivo* methods. Our results suggest that LDH leakage assay and MTT reduction test using cultured human fibroblasts could be predictive for the irritancy of various surfactants in human, and LDH assay is superior correlated with *in vivo* test (r=0.886) to MTT test with *in vivo* test (r=0.757).

Keywords □ human skin fibroblast; cellular toxicity; *in vivo* test; alternative method; surfactant

In vivo skin irritancy studies have been performed on animal models such as rabbit, guinea-pig or rat, and patch tests on human skin. The use of animals in screening and testing has been criticized by animal welfare groups. Experiments in human volunteers, on the other hand, are time consuming, expensive, and are impossible to perform directly without toxicological screening studies on the compounds. Hence, there has been much effort to seek and develop appropriate alternatives to *in vivo* tests (Hans *et al.*, 1994; William *et al.*, 1994).

However, there are currently no *in vitro* models that can provide data applicable to evaluate human skin irritation potentials (William *et al.*, 1994). Futhermore the European Union has already suggested that animal safety test on cosmetics should be performed until Jan. 1, 1998, if alternatives are available (Lee *et al.*, 1994). Therefore, simple and reproducible *in vitro* tests are required as screening procedures to estimate the skin irritancy of a large number of compounds.

The aims of this study are to investigate whether LDH leakage assay and MTT reduction test are sensitive methods of cytotoxicity test in cultured human skin fibroblast, and may be useful alternative model to human patch test, and rabbit intradermal safety test. Our studies were also performed to correlate *in vitro* with *in vivo* toxicity tests. This paper reports studies on surfactants selected as representative of known skin irritants.

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Materials and Methods

The following surfactants were tested both *in vitro* and *in vivo* method. Table I. List of tested surfactants.

Code	Name	Class
A	Tween 60	Nonionic
B	Triton X-100	Nonionic
C	α-Olefin sulfate	Anionic
D	Ammonium laureth sulfate	Anionic
E	Behenyl trimethyl ammonium chloride	Cationic
F	Distearyl dimethyl ammonium chloride	Cationic
G	Stearyl trimethyl ammonium chloride	Cationic
H	Benzalkonium chloride	Cationic
I	Cocoamidopropyl betaine	Amphoteric
J	Cocoamphocarboxyglycinate	Amphoteric

In vitro studies

Primary culture of human skin fibroblast

Human fibroblasts were isolated from circumcision discarded tissue. Separated dermis sheets were disso-

ciated into single cells by trypsin. Cells were maintained in minimum essential medium supplemented with nutrient mixture F-12, sodium bicarbonate and antibiotics (Park *et al.*, 1991).

Cytotoxicity experiments were initiated by plating 10^5 cells/m*l* into 24 well tissue culture plate for LDH assay and 96 well plate for MTT test. The plate was incubated for 24 hrs.

LDH Leakage assay

Quantitative measurement of LDH leakage into the culture medium after 3 hr incubation with surfactants was determined in order to evaluate cell membrane integrity. The average increasing rate of NADH was monitored using a LDH assay commercial kit (Sigma 228-20) as a percentage of total cellular LDH (Kodavanti *et al.*, 1993).

MTT test

MTT cytotoxicity test was conducted to assess mitochondrial integrity and cell viability. After 3 hr incubation with tested agents, medium was removed and 50 μl of MTT solution (2 mg/m*l*) was added to each well. At the end of 3 hr incubation period at 37℃, optical density of each well was measured at wavelength of 540 nm. Results were expressed as percentage of control (Grant and Acosta, 1994).

***In vivo* studies**

Human patch test

Volunteers aged between 20 and 35 years, who were not suspected of having any allergic contact dermatitis, were patch tested. Surfactants (1%) were applied with Haye's chambers on each volunteer's back. The test materials were removed after 2 days and the treated site was examined 30 min, 24 hr and 48 hr later. Reactions were scored according to the standard scoring system recommended by the ICDRG (International Contact Dermatitis Research Group) (Smiles and Pollack, 1977).

Intradermal safety test

Healthy rabbits were randomized and fur was removed from the area of the trunk. Each surfactants (1%) were intradermaly injected immediately after intravenous injection of 4 mg/kg of Evans blue solution (1%). The reaction was evaluated 1hr later after injection by measuring the zone of bluing with a millimeter ruler.

Statistical analysis

Log dose response curve parameter and EC_{50} values were determined using the Litchfield & Wilcoxon I methods. *In vivo* and *in vitro* values were correlated using the Spearman's rank correlation analysis.

Results

Cytotoxicity of surfactants *in vitro*

The effects of 10 selected surfactants on the cell membrane integrity and mitochondrial integrity were assessed using the LDH and MTT assay. The cells were exposed to a broad concentration range of each particular surfactant for 3 hr and then LDH and MTT assay were performed. The clear dose-response relationship was established for all test substances and the EC_{50} value of each surfactant (columns 1 & 2 of Table I.) was calculated from these dose-response plot. There was the increase in LDH leakage and decrease in MTT reduction which were concentration dependent for all tested surfactants. The results show that

Table II. Correlation coefficient between *in vivo* and *in vitro* methds

Method	LDH	MTT
Patch Test	0.886	0.753
Intradermal safety Test	0.700	0.553

The data was presented as Spearman's rank correlation coefficient

Table I. Cytotoxicity of surfactants on human fibroblast cells and their *in vivo* potential for skin irritation.

	LDH assay (EC_{50}, μg/m*l*)	MTT assay (EC_{50}, μg/m*l*)	Human Patch Test		Intradermal Injecton Test	
			Finding	Score	Finding	TIA
A	8920	1885	slight	0.42	non-irritant	0.5
B	97	707	mild	2.5	severe	5.0
C	24.3	129	severe	10.42	severe	3.8
D	16	38	severe	7.92	severe	5.0
E	33	33	mild	2.50	mild	2.8
F	943	313	mild	2.08	mild	2.0
G	8.2	38	severe	21.67	severe	5.8
H	23.4	13	severe	39.17	severe	5.8
I	35.1	58	severe	6.25	severe	5.3
J	76.7	462	mild	2.08	severe	4.5

TIA: Total injured area

LDH assay is more sensitive than MTT test.

Correlation between surfactants toxicity *in vitro* and *in vivo*

The cytotoxicity of surfactants *in vitro* systems were compared with their skin irritation potential in the Haye's chamber assay and rabbit intradermal safety test. Spearman's rank correlation coefficients are given in Table II. The closest relationship was found between LDH and human patch test ($r=0.886$). Apparently, correlation coefficient between MTT and in vivo patch test ($r=0.753$) was less than LDH assay. These results suggest that LDH assay is more pronounced method for these surfactants than MTT test.

Discussion

A large number of *in vitro* toxicological method have been proposed as alternatives to the *in vivo* test for skin irritation potentials (Hans *et al.*, 1994). These efforts have come to results although *in vitro* toxicity tests have not yet come to accept by the regulatory authorities.

LDH and MTT assay were used in this study because of possibility as alternatives *in vivo* test (Harvell *et al.*, 1994; Marinovich *et al.*, 1990) LDH assay is based on the principle that the lactate dehydrogenase is leaked from the cell by the treatment of surfactants. These mean that LDH assay evaluates the cell membrane integrity. MTT assay which evaluates mitochondrial dehydrogenase activity, could be used the detection of toxic effects, because plasma membrane and other cytoplasmic lipid bilayers such as mitochondrial membrane, could be impaired by surfactants. The correlation LDH with *in vivo* test was superior to that of MTT with *in vivo* test. In addition to, LDH was more sensitive than MTT test. These phenomenon could be explained that the LDH leakage, a nonspecific and highly sensitive marker of cell membrane damage, might provide a valid tool in evaluating the toxicity of new substances, particulary for compounds such as surfactants that interact with mucous membranes.

These results show that LDH leakage and MTT reduction assay give valuable informations about the relative potency of the compounds tested, and these variables could be used in the early stages of surfactant screening. The evaluation of LDH leakage assay allows the detection of toxic effects by surfactants that are ineffective on MTT reduction test.

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