In vitro cell recovery method as an alternative to human damaged skin recovery test

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Abstract

These days, the raw materials that have the cell recovering effect are used commonly in cosmetics. In this study, six materials were tested for the characteristics of recovering effect both *in vivo* and *in vitro*. Tested raw materials were Soypol, 3-APPA, Apple extract, *Polygonatum japonicum* extract, *Scutellaria baicalensis* extract, Aloe extract. Among these materials, Soypol and 3-APPA were synthesized and others were made by extraction at the Pacific R&D Center. Human forearm skin and cultured skin cells were damaged by sodium lauryl sulfate (SLS) and then raw materials were applied for open treatment on SLS damaged human skin or cells.

The recovering effects of raw materials *in vivo* were evaluated by measuring transepidermal water loss (TEWL), skin hydration and erythema and *in vitro* effects of proliferating cells were assessed by neutral red uptake assay. In the *in vivo* study, only the evaluation by TEWL showed correlation with the visual score. Out of six materials, 3-APPA had the most positive effect in both *in vitro* and *in vivo* studies and the correlation was $r = 0.8286$ ($p = 0.042$).

Introduction

Skin is a complex, organized structure and a living "cloth" covering the interior body and the barrier function of skin resides mostly in the epidermis.[2,12] Skin has a great ability of regeneration and the structural and functional integrity of epidermis are maintained by continuous proliferation and differentiation of cells. Increasing proliferation activity in epidermal basal cells is very important in the recovery of damaged skin.

In today's stressful environment such as rapid temperature changes and contact with chemicals, human skin surface is subject to continuous damages. Cosmetologists are preserving their efforts to recover such damages through cosmetics. The materials that have cell regenerating activities are popular ingredients in cosmetics.

Sodium lauryl sulfate (SLS) is an anionic surfactant used as an emulsifier in many pharmaceutical vehicles, cosmetics, foaming dentifrices, and even food. The chemical formula fo sodium salt of lauryl sulfate is: $CH_3(CH_2)_{10}CH_2OSO_4Na$.[10] The action of SLS on surface tension is putatively the cause of its irritancy, and its great capacity for altering the stratum corneum makes it useful for enhancing the penetration of other substances in patch tests and animal assays.[10,14] Therefore the induction of surfactant-induced irritation has been extensively studied in recent years. Although the mechanism is unknown, SLS has been used extensively as a model irritant in the field of irritant skin testing.[1,5,9]

In this study, sodium lauryl sulfate (SLS) skin damage model which is primarily used in *in vivo* tests was applied to cell cultures and the efficacies of the raw materials that are presumed to have the recovering effects were tested. And then the *in vivo* and *in vitro* tests were compared in order to investigate the usefulness of *in vitro* method as a prescreening method.

Materials and Methods

1. Experimental reagents and equipment

SLS and neutral red were obtained from Sigma (USA) and the cell culture medium and other reagent were obtained from Gibco (USA).

Test materials were Soypol, 3-APPA, apple extract, Polygonatum japonicum extract, Scutellaria baicalensis extract and Aloe extract. Among these materials, Soypol and 3-APPA were synthesized and others were made

by extraction (Table 1) at the Pacific R&D Center.

The absorbance was measured by microplate reader El 310 (Bio-Tek). Transepidermal water loss (TEWL), skin color and hydration were measured by evaporimeter EPI (Servo Med, Sweden), Chromameter CM1000 (Minolta, USA) and Corneometer CM 820 (CK, Germany) respectively.

2. *In vitro* cell recovery test

1) Cell culture: Normal human keratinocyte was obtained from human foreskin tissues. Keratinocytes were cultured in DMEM media containing 10% heat inactivated fetal bovine serum, 100 IU penicillin G/ml and 100 µg streptomycin sulfate/ml.

The cells were grown on 75 cm^2 flasks and subcultured on a 96-well flat-bottomed microplate for use in testing. The cells were incubated at 37°C in 95% air, 5% CO_2.

2) Determination of SLS concentration: To determine the SLS concentration for reducing cell viability to 50%, various concentrations of SLS were added to the medium. Cell viability was measured by neutral red uptake[4] assay after incubating for 24 hrs. The empirically determined concentration was used to induce the damage to the cells.

3) In vitro cell recovery test: For the cell recovery test, cells were seeded in 96-well microplates (1×10^4 keratinocytes per well in 200 µl complete medium). The cells were preincubated in DMEM with 10% FBS in a humidified atmosphere with 5% CO_2 at 37°C for 24 hrs. The cells were then exposed to media containing SLS and 2% FBS for 24 hrs and the medium was removed. The cells were then exposed to new media containing test materials and 2% FBS using double dilution method. At this point, 8 wells were not treated with test materials as a control to calculate the cell viability. After 48 hr incubation, the medium was removed.

4) Neutral red uptake assay: NR solution was prepared 1 day in advance, by adding the NR of 50 µg/ml final concentration to the complete medium. It was incubated overnight at 37°C and the precipitated crystal dye was removed by centrifugation (10 min at 1500 *g*). 200 µl NR medium was added to each well and incubated for at 37°C 3 hrs. Added NR medium was again removed and cells were washed quickly with the wash/fix solution of 1% formaldehyde and 1% $CaCl_2$. Medium was then aspirated and 200 µl solvent solution (1% acetic acid + 50% ethanol) was added. The plates were then shaken rapidly for 20 min. Plates were read at 540 nm on a microplate reader EL 310 (Bio-Tek).

3. Damaged skin recovery test

1) Determination of optimum condition for skin damage: To determined the optimum condition for skin damage, 40 volunteers were tested under three different conditions (5% SLS 2 hr patch, 1% SLS 24 hr patch, 0.5% SLS 24 hr patch) using a 18 mm Hilltop chamber containing 150 µl SLS solution and Micropore tape (3M, USA).

2) Damaged skin recovery test: 5 male volunteers between the ages of 27-30 years with healthy skin were included in the test. Measurements were carried out at a temperature of 25°C and a relative humidity of 60%. Subjects were accustomed to ambient conditions for 20 min prior to any measurement. The test was carried out on both volar forearms. Initially untreated skin was measured in all eight areas to find baseline values. After measuring, the Hilltop chamber containing 150 µl 0.5% SLS sol. was appled to the test site and occluded with micropore tape. After the 24 hr occlusion, chambers were removed and test sites were washed with warm water. After 1 hr, each test site was measured and about 100 µl of solubilized test materials and distilled water (vehicle) were applied on them with one site remained untreated as a control. In the following 3 days, test materials were applied twice daily.

Measurements were evaluated every morning before the first application. Before measurement, forearms were washed with running water.

4. Statistics

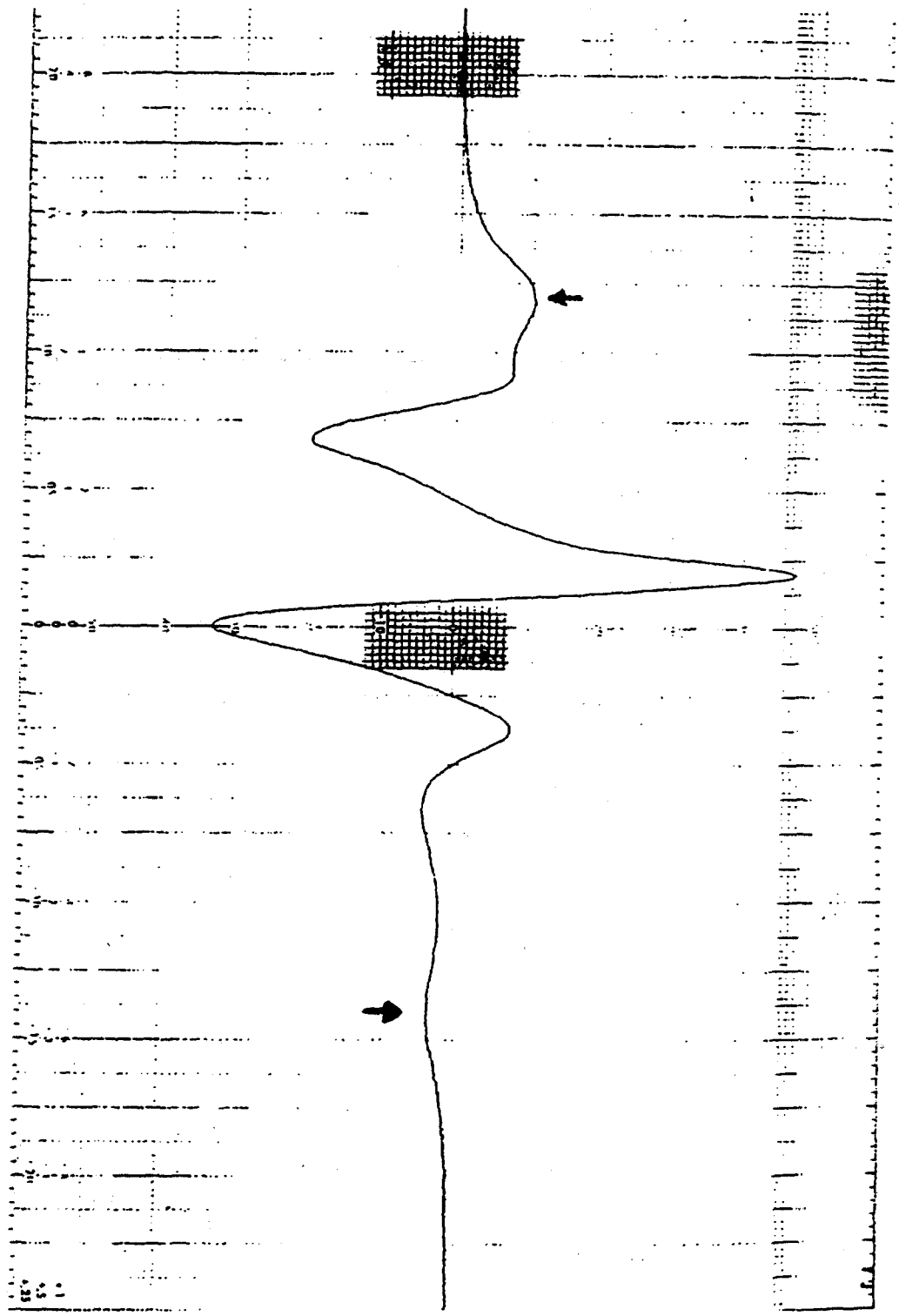
Using the computer program (SPSS version 6.0), data were statistically analyzed. Differences between the treatment groups were tested for their statistical significance by the One-way ANOVA test and multiple comparisons of all groups were performed by the LSD procedure. *In vivo* and *in vitro* values were correlated using the Spearman's rank correlation analysis.

Results

1. *In vitro* cell recovery test

The SLS concentrations reducing the cell viability to 50% was 0.0025% (Fig. 1). Since it was previously observed in my laboratory that most cells recovered normal morphology 48 hrs after removing SLS media, cells were treated with test materials for 48 hrs after SLS treatment.

The results are summarized in table 2 and among the six materials, 3-APPA showed the best proliferation effect.



(A) SOY LECITHIN LIPOSOMES

Table 1. Test materials listing

No.	*Test materials*	*Specificity*	*Type*	*Source*	*Maker*
1	Soypol	Glutamic acid polymer + Fructose polymer	Powder	Fermentation	Pacific R&D
2	3-APPA	3-amino propane phosphoric acid	Powder	Synthesis	"
3	Apple extract	Water extraction	Liquid	Plant	"
4	*Polugonatum japonicum* extract	Water extraction	Liquid	Plant	"
5	*Scutellaria baicalensis* extract	Water extraction	Liquid	Plant	"
6	Aloe extract	Water extraction	Liquid	Plant	"

* Test materials are not containing any preservatives.

Table 2. Summary of in vitro cell recovery test results

No.	*Test materials*	*Cell viability (%)*[a]	*Concentration (%)*
1	Soypol	114.24 ± 2.83[b]	0.0039-0.0078
2	3-APPA	119.51 ± 2.54[c]	0.0078-0.0156
3	Apple extract	105.07 ± 5.93	0.0782-0.1563
4	*Polugonatum japonicum* extract	108.17 ± 6.31	0.1563-0.3125
5	*Scutellaria baicalensis* extract	111.81 ± 7.16	0.0782-0.1563
6	Aloe extract	111.89 ± 3.04	0.0782-0.1563
7	Bovine fetal serum	126.34 ± 6.56[d]	10

[a] Each materials were tested 4 times and cell viability(%) was averaged.
[b] It is significantly different from Apple extract.
[c] It is significantly different from Apple extract, *Polugonatum japonicum* extract, *Scutellaria baicalensis* extract and Aloe extract .
[d] It is significantly different from all other materials.

Table 3. Determination of test condition (n=40)

	Condition 1	*Condition 2*	*Condition 3*
	5% SLS 2hr patch	1% SLS 24 hr patch	0.5% SLS 24 hr patch
TEWL (Mean±SD)	11.20 ± 4.58	24.95 ± 14.89	14.29 ± 3.68
Clinical score	+	++	+

Table 4. Summary of damaged skin recovery test results: Change of recovery rate (TEWL) after treatment (n=5)

No.	*Test materials (Conc. %)*	*Day 1 TEWL Recovery rate (%)*	*Day 2 TEWL Recovery rate (%)*	*Day 3 TEWL Recovery rate (%)*	*Visual score*[a]
1	Soypol (10%)	1.7718±26.6477	25.4460±32.3022	40.3106± 9.6755	2.30±0.89
2	3-APPA (10%)	5.3993±25.7970	39.6834±16.2474	47.5738± 8.8665	2.20±0.89
3	Apple extract (100%)	-1.0638± 5.1410	11.7390±20.7369	36.8754± 8.4559	6.20±1.30
4	*Polugonatum japonicum* extract (100%)	2.2917±25.1541	20.4624±25.9761	33.3410±13.6232	6.00±1.22
5	*Scutellaria baicalensis* extract (100%)	8.7748±13.5199	9.5892± 4.2884	37.5299± 6.5759	3.10±0.84
6	Aloe extract (100%)	-3.6408±19.4656	7.2664±17.8214	38.6454± 7.2935	2.40±0.89
7	D.W. (vehicle)	-12.6324±47.6763	18.3480±25.6669	30.4802± 7.0123	6.60±0.55
8	No treatment	-11.1366±47.7982	16.9378±24.6394	31.7990± 8.8928	7.20±1.30

[a] Each value is the mean of ranks on 5 subjects