

Evaluation of human skin irritation by carboxylic acids, alcohols, esters and aldehydes, with nitrocellulose-replica method and closed patch testing

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Closed patch testing and the nitrocellulose-replica method are performed as useful clinical methods for the evaluation of human skin irritation by cosmetics and topical medicaments. Comparison of the sensitivity between microscopic scoring by nitrocellulose-replica method and visual scoring by closed patch test in the detection of skin irritation, however, has not been well studied with statistical analysis. Here, we evaluated human skin irritation by carboxylic acids, alcohols, esters and aldehydes, with different chain length (C8-C18), using both methods. The results of closed patch testing showed that, although the score of skin irritation for carboxylic acids (C8, C12), alcohols (C8) and aldehydes (C8), tested at a concentration of 0.5 m-2.0 m, significantly increased with increasing concentration of the test compounds, ester compounds scarcely caused any irritation on the surface of the skin occluded. In addition, an increase of carbon chain length in the test compounds made it impossible to detect skin irritation. In contrast, the nitrocellulose-replica method could evaluate skin reactions against very weak irritants that gave no macroscopic alterations on the skin surface in the closed patch test. However, the scoring system is somewhat subjective and should be improved to make the analysis more objective.

Key words: skin irritation; nitrocellulose-replica method; closed patch test; carboxylic acid; alcohol; ester; aldehyde; semi-open; occlusion. © Munksgaard, 1996.

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Closed patch testing is performed worldwide as a clinical evaluation for the detection of human skin irritation by cosmetics, topical medicaments, and their materials. The test is capable of evaluating skin irritation with macroscopic observation of inflammatory alterations, such as redness and swelling, on the areas of occluded skin with irritants (1).

On the other hand, Kawai et al. (2) have recently found that the nitrocellulose-replica method is a very sensitive skin test against weak irritants, compared to evaluation by the closed patch test. While no visual alterations are found on the skin surface applied with commercial products, the replica method can detect several morphological changes of the surface microscopically (3). In addition,

since test compounds are applied on the skin surface semi-open in the replica method, such semi-open application is similar to the practical use of cosmetics and topical medicaments, compared to the closed patch test. In Japan, the replica method is officially used as one of the evaluations of skin irritation. Evaluation of skin irritation by approximately 11,000 commercial products (e.g., cosmetics, topical medicaments, textiles, and softeners and pigments for textiles) with the replica method has, so far, correlated well with the frequency of claims of skin damage by these products (4).

In the present paper, we investigated the sensitivity of microscopic observation by the nitrocellulose-replica method and visual evaluation by the closed patch test in the detection of human skin

irritation by carboxylic acids, alcohols, esters and aldehydes, with different carbon chain length (C8-C18), as test compounds.

Materials and Methods

Test compounds

Each test compound was adjusted to a concentration (molality) of 0.5 *m* to 2.0 *m* by using petrolatum (Nikko SC Corporation, Japan) as vehicle, as shown in Table 1. Test compound nos. 1-8 and 12, nos. 9-11, and no. 13 were purchased from Nakalai Tesque Inc. (Kyoto, Japan), Wako Pure Chemical Industries Ltd. (Osaka, Japan), and Givaudan-Roure (Paris, France), respectively.

Subjects

20 healthy volunteers, aged 26-29 years, entered the present study after signing a written consent form.

Nitrocellulose-replica method

The nitrocellulose-replica method is a useful test for evaluating skin irritation by microscopic observation of replicas taken from the skin surface applied with test compounds. Application of the test compounds on the skin surface and preparation of the replicas were performed according to the procedure described previously (1, 2). Briefly, each compound was applied on the flexor side of both upper arms with semi-open patch test and was then removed after 24 h of application. At 30 min after removal, visual evaluation of the semi-opened skin was performed and then replicas of the skin surface were taken. Nitrocellulose disks were

softened with *n*-butyl acetate and were then carefully put on the skin surface where the compounds had been applied. After 1-2 min, the disks were gently removed from the skin and attached to mounts. The specimens obtained were observed under a microscope with 50× magnification.

Closed patch test

15 μ l aqueous of test compound was applied to a filter disk, which was immediately fitted into Finn Chambers on Scanpor tape (Epitest Ltd Oy, Finland). The test was performed on the upper back of subjects. The Finn Chambers were removed after 24 h of application.

Scoring system

In the nitrocellulose-replica method, morphological changes on the surface of skin applied with each compound could be detected. Normal skin shows thin lines along skin furrows, whereas the abnormal region shows wide translucent bands which indicate deepened skin furrows (2). Although other abnormal changes, including loss of triangular configuration of skin furrow pattern or membranous desquamation, can be observed microscopically on the skin surface applied with strong irritants, only the appearance of deepened skin furrows was detected in this study. Therefore, microscopic observation was expediently scored for the appearance of deepened skin furrows on replicas: no skin furrow change scored 0, skin furrows which were deepened in up to 25% of the area of the sample scored 0.5, from 25% to 50% scored 1.0, and for more than 50% scored 2.0. Observation of replicas was performed by 2 persons and variation in the skin irritation scores obtained was below $\pm 5\%$.

In the closed patch test, skin reactions were evaluated with visual scoring of occluded skin with test compounds. Although visual observation was performed at 1 h and 24 h after removal of the compounds, there was basically no difference between skin irritant tendencies of results obtained at both observation times. Therefore, the score at 1 h after removal was adopted in this study. Macroscopic observation was scored for erythema: no reaction 0, slight redness 0.5, moderate redness 1.0, and intense redness 2.0.

Statistical analysis

To compare the dependence on the concentration and carbon chain length of test compounds of skin irritation, data were analyzed by Wilcoxon's rank-sum test. $p < 0.05$ was chosen as the level of statistical significance.

Table 1. Test compounds and purity

No	Test compounds	Purity (%)
Carboxylic acids		
1	caprylic acids (C8)	98
2	lauric acid (C12)	99
3	palmitic acid (C16)	99
4	stearic acid (C18)	98
Alcohols		
5	octyl alcohol (C8)	99
6	lauryl alcohol (C12)	99
7	cetyl alcohol (C16)	96
8	stearyl alcohol (C18)	95
Esters		
9	methyl n-octanoate (C8)	98
10	methyl laurate (C12)	98
11	methyl palmitate (C16)	95
Aldehydes		
12	octyl aldehyde (C8)	95
13	lauryl aldehyde (C12)	99

Results

Both microscopic and visual scores of human skin irritation by carboxylic acids, alcohols, esters and aldehydes are shown in Fig. 1. In Tables 2 and 3, the effects of concentration and carbon chain length of test compounds on skin irritation evaluated with nitrocellulose-replica method and closed patch test were statistically analyzed. The score of each test compound was subtracted from that of petrolatum used as a control, since the concentration of the test compound was adjusted by using petrolatum. In the present study, the irritation scores for petrolatum in nitrocellulose-replica method and closed patch test were 0.5–0.7 and 0, respectively.

Carboxylic acids

The results evaluated with closed patch testing show that the score of skin irritation for caprylic acid (C8) and lauric acid (C12) significantly in-

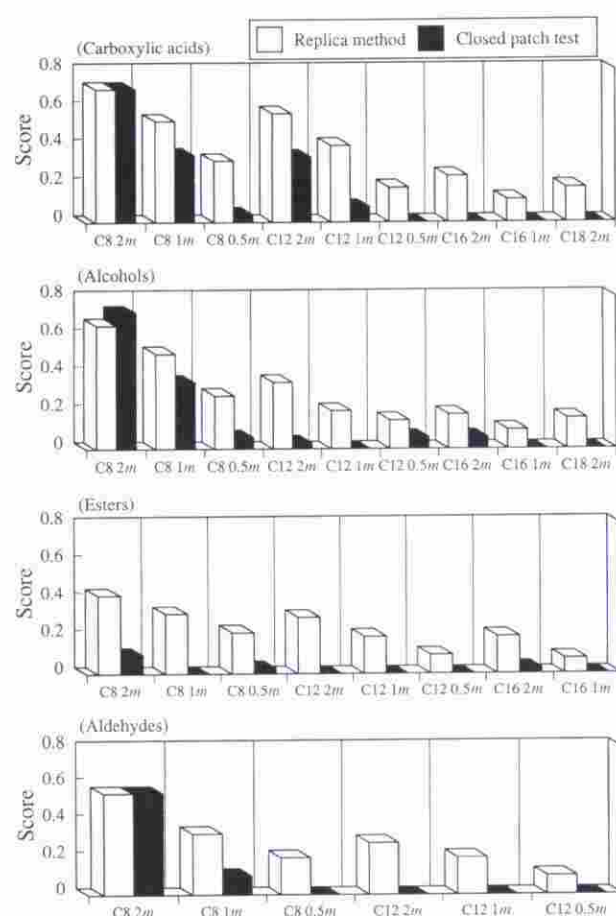


Fig. 1. Evaluation of human skin irritation by carboxylic acids, alcohols, esters and aldehydes, with nitrocellulose-replica method and closed patch testing. All data represent averages of $n=20$.

Table 2. Effect of concentration of test compounds on human skin irritation evaluated with nitrocellulose-replica method and closed patch testing

Test compounds	Evaluation	
	(replica)	(closed)
Carboxylic acids		
C8	2 $m>0.5 m$	2 $m>1 m>0.5 m$
C12	2 $m>0.5 m$	NS
C16	2 $m>1.0 m$	NS
C18	—	—
Alcohols		
C8	2 $m>0.5 m$	2 $m>1 m>0.5 m$
C12	2 $m>0.5 m$	2 $m>1 m, 0.5 m$
C16	NS	NS
C18	—	—
Esters		
C8	2 $m>0.5 m$	NS
C12	NS	NS
C16	NS	NS
Aldehydes		
C8	2 $m>0.5 m$	2 $m>1 m>0.5 m$
C12	NS	NS

>: statistical significance ($p<0.05$) NS: no significance —: no comparison.

Table 3. Effect of carbon chain length of test compounds on human skin irritation evaluated with nitrocellulose-replica method and closed patch testing

Test compounds	Evaluation	
	(replica)	(closed)
Carboxylic acids		
2 m	C8>C12, C16, C18	C8>C12, C16, C18
1 m	C8>C16	C8>C12, C16
0.5 m	NS	NS
Alcohols		
2 m	C8, C12>C16	C8>C12>C16, C18
1 m	C8<C16	C8<C12, C16
0.5 m	NS	NS
Esters		
2 m	C8>C16	NS
1 m	NS	NS
0.5 m	NS	NS
Aldehydes		
2 m	NS	C8>C12
1 m	NS	C8>C12
0.5 m	NS	NS

>: statistical significance ($p<0.05$) NS: no significance.

creased with increasing concentration of the test compound. With palmitic acid (C16) and stearic acid (C18) having a longer chain length, however, almost no concentration dependence was seen, because of a dramatic decrease in the score of skin irritation. Although a similar concentration dependence profile was obtained in experiments using the nitrocellulose-replica method, the method could detect skin reactions against C16

and C18 that gave no macroscopic changes in closed patch testing. Moreover, the data of both evaluation tests indicate that a decrease in carbon chain length of test compounds at a concentration of 1 *m* and 2 *m* led to an increase in the irritation score.

Alcohols

Although the irritation score for octyl alcohol (C8) in a closed patch test significantly increased with increasing concentration of the test compound, lauryl alcohol (C12), cetyl alcohol (16), and stearyl alcohol (C18) gave a low irritant score regardless of the concentration. On the other hand, the nitrocellulose-replica method could detect skin irritation against C12, C16 and C18 at all concentrations tested. The irritation scores of 2 *m* C12, C16, and C18 alcohols were significantly higher than those of 0.5 *m* and 1 *m*, respectively. The data of both the replica method and closed patch test indicate that a decrease of carbon chain length in test compounds, except for 0.5 *m*, led to an increase in the score of skin irritation.

Esters

Since each irritation score of methyl *n*-octanoate (C8), methyl laurate (C12), or methyl palmitate (C16) was very low in a closed patch test, it was impossible to compare the degree of skin irritation caused by their compounds. However, in the replica method, the skin irritation tended to be multiplied with increasing concentration of the compounds. In addition, the results of the replica method show that shorter chain length of esters resulted in higher irritation score. For example, the irritation score of C8 at a concentration of 2 *m* was significantly higher than that of C16 at the same concentration.

Aldehydes

In a closed patch test, an increase in irritation score of octyl aldehyde (C8) was clearly dependent on higher concentration, but all concentrations of lauryl aldehyde (C12) did not cause any skin irritation at all. On the other hand, the results of the replica method show that the scores of both C8 and C12 compounds have a tendency to increase with increasing concentration. With respect to the effect of carbon chain length on skin irritation, evaluation with the closed patch test indicates that a decrease of carbon chain length in C8 and C12 compounds, at a concentration of 1 *m* and 2 *m*, induced a significant increase in the irritation score. However, as long as skin irritation was

evaluated with the replica method, the chain length dependence was not confirmed statistically.

Discussion

We compared the nitrocellulose-replica method with closed patch testing, in the evaluation of skin irritation by carboxylic acids, alcohols, esters and aldehydes. Ray et al. (5) demonstrated that C8, C10, and C12 free fatty acids were significantly more irritating than C2, C4, C6, C14 and C16, as a result of skin irritant evaluation after the even-numbered free fatty acids from C2 to C16 were applied daily under occlusive patch test to human skin until detectable erythema appeared. A similar result was reported by Stillman et al. using saturated free fatty acids from C3 to C18 and unsaturated C18 free fatty acids (6). In the present paper, we have also obtained evidence that, in a closed patch test, C8 compounds (caprylic acid, octyl alcohol and octyl aldehyde) among carboxylic acids, alcohols and aldehydes, except for esters that scarcely caused any skin irritation, were significantly more irritating than those of other even-carbon chain length (C12-C18). Moreover, that data of the closed patch test showed that lower concentration or longer chain length of test compounds resulted in a dramatic decrease in the irritation score.

In contrast, the nitrocellulose-replica method made it possible to detect skin reactions, with concentration dependence, against very weak irritants (all ester compounds, carboxylic acids of C16 and C18, alcohols of C12 to C18 and lauryl aldehyde) that gave no macroscopic alterations in the closed patch test. Kawai et al. (2) have recently reported that, using sodium lauryl sulfate as a skin irritant, the nitrocellulose-replica method, which can observe several morphological changes on the skin surface applied with irritants, is a more sensitive skin test against weak irritants than the closed patch test. Our results have also demonstrated that, although no macroscopic alterations were found on the areas of semi-opened skin with some weak irritants described above, the appearance of deepened skin furrows could be observed as skin irritation microscopically. Although 2 skilled observers evaluated skin irritation in the nitrocellulose-replica method, the scoring system may be somewhat subjective. We are currently developing the scoring system, using computer image analysis, to quantify the irritant response more objectively.

Taken together, the nitrocellulose-replica can evaluate skin reactions against weak irritants that give no visual changes in a closed patch test. This fact indicates that evaluation only with the

closed patch test may be insufficient to determine whether or not a compound causes skin irritation.

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