

## The Application of Nanoliposome Composed of Ceramide as an Anti-irritant in Cosmetics

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The objective of this study is to suggest the potentialities of nanoliposome composed of ceramide as an anti-irritant against various irritants used in cosmetics. Ceramides are major structural components of the epidermal permeability barrier, which is known to play an essential part in human physiology by not only preventing the loss of water from the body but also protecting the body from external physical, chemical, and microbial insults. According to the results, better effects on reinforcement of skin barrier function and anti-irritation were obtained with nanoliposome composed of ceramide than with dispersed ceramide. And, we performed *in vitro* skin penetration test using horizontal Franz diffusion cells with skin membrane prepared from hairless mouse to evaluate the influence of nanoliposome composed of ceramide on the skin penetration of lactic acid in formulations. From the results, we found that the anti-irritation effects of nanoliposome containing ceramide were due to reduced penetration rate of irritants. Conclusively, we could develop a new anti-irritation system and apply this nanoliposome composed of ceramide to the final cosmetic products successfully.

**Key words** – nanoliposome, ceramide, anti-irritation, skin barrier function

It has been known for many years that the stratum corneum (SC) is of great importance for proper skin barrier function. This uppermost layer of the epidermis protects the human body against the loss of physiologically important components and against potentially damaging environmental insults[1]. The stratum corneum is composed of two major components: corneocytes and intercellular lipids. By the "brick and mortar model", which is one of the most commonly employed analogies for describing SC organization, the hydrophilic corneocyte (brick) fills the major volume of the wall (SC) surrounded by the hydrophobic intercellular lipid (mortar); the most dominant part of the epidermal permeability barrier function seems to be presented by the intercellular bilayers which are enriched in cholesterol, ceramides, and free fatty acids. These lipids are delivered to the intercellular spaces as a mixture of precursors by the secretion of epidermal lamellar body contents. Following their secretion, the lipid precursors are metabolized within the extracellular spaces by colocalized, LB-derived hydrolytic enzymes into hydrophobic, lamellar basic unit structures, which mediate barrier function[2,12]. Many different groups of investigators have demonstrated that there is a marked

decrease in ceramide level within the stratum corneum of patients involving skin barrier abnormalities such as atopic dermatitis and irritant/allergic contact dermatitis[14]. As ceramides are an important determinant involved in water-holding properties[6] and stratum corneum barrier function [5,10], the insufficiency of ceramides may provide an etiological basis for dry and barrier-disrupted skin. For this reason, topical treatment with ceramides has been comprehensively used for skin disorders such as psoriasis, atopic dermatitis, irritant contact dermatitis, allergic contact dermatitis, and so on. However, there have been few reports describing the possibilities of these structural lipids as anti-irritants in cosmetics. In this study we compared the anti-irritation effects of formulas containing nanoliposome composed of ceramide with those containing dispersed ceramide and placebo. To investigate the potency of nanoliposome composed of ceramide for reinforcement of skin barrier function, we examined the recovery rate of transepidermal water loss (TEWL) after tape stripping and the inhibitory effect of flush induced by methyl nicotinate. And, clinical studies such as human patch test and stinging potential test were conducted to evaluate the anti-irritation effects of nanoliposome composed of ceramide using lactic acid as a chemical insult. Finally, we performed *in vitro* skin penetration test using horizontal Franz diffusion cells with skin membrane prepared from hairless mouse to evaluate the influence of

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nanoliposome composed of ceramide on the skin penetration of lactic acid in formulations.

## Materials and Methods

### Preparation of nanoliposome composed of ceramide and formulas used in these experiments

Nanoliposome composed of ceramide was made from a mixture of glycerin (65.0 wt%), caprylic / capric triglyceride (12.0 wt%), phospholipid (5.0 wt%), ceramide III (8.0 wt%) and ionized water (10.0 wt%) at 85°C. The ceramide III was purchased from Doosan (Korea) and phospholipid (Lipoid S75-3) was from Lipoids (Germany), who provided us with the following lecithin specification: phosphatidylcholine content 67.0~71.0 wt%, lysophosphatidylcholine content 2.3 wt%, phosphorous content 3.4~3.7 wt%, water and other remaining content ≤3.0 wt%. In order to reduce the particle size of liposome to nanometer range, high pressure homogenization was performed with a microfluidizer. The mixture was pumped through microchannel, called as an interaction chamber of a M-100F microfluidizer (Microfluidics Corp., U.S.A) at a pressure of 1,000 bar. This process was repeated up to 3 times. The average particle size of nanoliposome composed of ceramide was about 300~600 nm. We manufactured some formulas (Table 1) containing nanoliposome composed of ceramide or dispersed ceramide III to assess its effects on reinforcement of skin barrier function, anti-irritation activity against irritant used in cosmetics.

### TEWL measurements

TEWL is a sensitive index of skin barrier integrity. This parameter evaluates the water loss in g/m<sup>2</sup>/h, measured using a Tewameter TM 210 (Courage & Khazaka, Germany). A total of 10 healthy volunteers without past or present of skin diseases (1 male, 9 females, mean age 26 years, range 23~30) were included in the study. Written informed consent was obtained from all participants. Measurements were performed, according to the guidelines from the standardization group of contact dermatitis[11], at time 0-before (baseline TEWL) and at just after tape stripping. For tape stripping, adhesive tapes (3M) were applied with gentle pressure to the test areas of forearm and then removed. This procedure was repeated until the skin was glistening. And another measurements were taken more than 4 h after application of the formulas.

### *In vivo* methyl nicotinate assay

It is known that topically applied methyl nicotinate leads to a generalized cutaneous erythema (flush) mediated at least in part by prostaglandin biosynthesis[13]. Ten healthy volunteers aged 23~30 (1 male, 9 females, mean age 26 years) were included in the study. They did not receive any anti-inflammatory drug for 10 days prior to the study. There were four test areas per volunteers. Each area received either one of the three tested formulas (Formula #1~#3); the fourth was left untreated. Prior to the application of test materials, we measured the baseline  $a^*$ -value

Table 1. Formulas used in these experiments.

Raw materials	Dosage (% w/w)						
	#1	#2	#3	#4	#5	#6	#7
Caprylic/Capric triglyceride	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Mineral oil	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Cetearyl alcohol & Cetearyl glucoside	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Glyceryl stearate & PEG-100 stearate	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Polyacrylamide & C13-14 Isoparaffin & Laureth-7	0.4	0.4	0.4	0.8	0.8	0.8	0.8
Methylparaben	0.2	0.2	0.2	-	-	-	-
Sodium lactate (60.0%)	-	-	-	2.0	2.0	2.0	2.0
Potassium hydroxide (10.0%)	-	-	-	-	7.0	7.0	7.0
Lactic acid	-	-	-	-	5.0	5.0	5.0
Ceramide III	-	-	-	-	-	2.0	-
Nanoliposome composed of ceramide	-	1.0	12.5	-	-	-	25.0
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Formula #1~#3 were used in the tests for examination of the recovery rate of TEWL after tape stripping and the inhibitory effect of flush induced by methyl nicotinate. And Formula #4~#7 was used in the tests to compare the anti-irritation effects of formulas containing nanoliposome composed of ceramide with those containing dispersed ceramide and placebo (formula #4: negative control which did not contain both irritant and anti-irritant, formula #5: positive control which only contained irritant).

(red-green axis) using a minolta cromameter Type CR-200 (Minolta, Osaka, Japan). And then, the formulas were applied for 4 h on the subjects' forearms in a randomized single-blind manner. At the end of the application time, the test materials were washed out, and the subjects allowed to sit at rest. Thirty minutes later, a filter paper saturated with an aqueous solution of 3mM methyl nicotinate (20  $\mu$ l) was applied to each test site for 1 min 30 sec. After 30 min, the vascular responses to methyl nicotinate (i.e. increases in flush) were then simultaneously measured by using a minolta cromameter Type CR-200.

#### Human patch test for assessing primary skin irritation

We performed human patch test to evaluate primary skin irritation potential according to the method of CTFA safety testing guidelines[15] with a slight modification. Twenty human volunteers (eight women and twelve men), free from skin disease or allergy, participated in the test. Their age ranged 23 to 35 years (average 28). Occlusive patches were applied to suitable areas of skin on the forearm using Finn chambers (Epitest Ltd Oy, Finland) attached to scanpor tape after cleansing and drying the test sites with 70% ethanol. The patches were removed after 24 hours, and the test sites were assessed for the presence of irritation at 30 minutes and 24 hours following patch removal, according to the standards of International Contact Dermatitis Research Group. The evaluation of skin irritation potency is presented as a mean score from the following equation[3,8]:

$$\text{Mean Score} = \frac{\text{Grade} \times \text{No. of responses} \times 100 \times 1/3}{3(\text{Maximum grade}) \times 20(\text{Total subjects})}$$

Grade Score	:	No reaction
- 0	:	Weak positive reaction (erythema)
± 0.5	:	Moderate positive reaction (erythema)
+ 1	:	Strong positive reaction (erythema, edema)
++ 2	:	Severe positive reaction (erythema, edema,
+++ 3	:	vesicles)

#### Stinging potential test for assessing subjective irritation

As originally published, the human subjective (sensory) irritation assay required the use of 110°F environmental chambers with 80% relative humidity[4]. And other investigators used a 15 min treatment with a commercial facial sauna to produce facial sweating[9].

Volunteers were sufficiently sweated by treating hot steam of steam generator (Vapozone 707, Taiwan) for 15 min. Sweat was removed from nasolabial fold and cheek. A 5%

aqueous solution of lactic acid was then rubbed briskly over the area. Those who reported stinging for 3 to 5 min within the first 8 min were designated as stingers and used for subsequent tests. Twenty human volunteers (ten women and ten men) participated in this test as stingers. Their age ranged 23 to 32 years (average 28). Subjects were asked to evaluate the degree of subjective irritation (stinging, itching, burning, etc.) as 0=no sensation, 1=slight sensation, 2=moderate sensation, 3=severe sensation. The subjective irritation was evaluated 10 sec and 2.5, 5, 8 min after application of test material. The evaluation of subjective irritation potency is presented as a response rate (%) from the following equation:

$$\text{Response rate (\%)} = \frac{\text{Response score} \times 100}{\text{Maximum score (30} \times \text{total subjects} \times \text{No. of response)}}$$

#### In vitro skin penetration test

The influences of nonliposome composed of ceramide or dispersed ceramide on the penetration of irritant such as lactic acid from the formulas through hairless mouse skin (5~8 week old, female, abdominal skin) were studied with home-made, glass, vertical Franz diffusion cells (Labfine Co.)[7]. In these cells, the skin membranes were sandwiched between the upper donor compartment and the lower receptor compartment. The circular area of skin that was in contact with the two compartments was 0.64 cm<sup>2</sup>. The receptor compartment (5 ml) was filled with 33 mM phosphate buffered saline (pH 7.4). The receptor solution was maintained at 37°C and stirred with a magnetic stirrer at 600 rpm. Usually, 0.5 ml of formulas was placed in the donor compartment onto the skin, and the concentration of test material in 100  $\mu$ l of receptor solution was determined by high performance liquid chromatography (Waters Alliance 2695 system, U.S.A.) equipped with an UV detector (Waters Alliance 2487 UV detector, U.S.A.). The UV detector was set at 210 nm. A Mightsil C18 column (250 mm length, 4.6 mm i.d.) from kanto chemical (Japan) was used. Elution was performed with 25 mM KH<sub>2</sub>PO<sub>4</sub> (pH 2.5) at a flow rate of 0.7 ml/min. Quantifications were performed by comparing peak heights with calibration curves obtained with known amounts of materials under identical analytical conditions.

## Results and Discussion

#### Recovery rate of TEWL after tape stripping

First of all, we investigated the potency of nanoliposome

composed of ceramide for reinforcement of skin barrier function by measuring the recovery rate of TEWL after tape stripping. As shown in Fig. 1, the mean TEWLs of 40 test areas (4 sites per volunteer) before (baseline) and after tape stripping were about 9.04 and 19.58 g/m<sup>2</sup>/h, respectively. And we observed that the treatment with formula #3 containing nanoliposome composed of ceramide significantly accelerated repair of skin barrier damage in comparison to the untreated site, formula #1 (placebo) and formula #2 containing dispersed ceramide.

**Inhibitory effect of flush induced by methyl nicotinate**

It is known that topically applied methyl nicotinate penetrates rapidly through the stratum corneum, reaches the dermal capillaries and exerts its pharmacological action via an erythematous reaction. Therefore, the inhibition effect of flush induced by methyl nicotinate could be thought that was mediated at least in part by the ability of nanoliposome composed of ceramide to reinforce the skin barrier function. According to our results, presented in Fig. 2 and Table 2, a significant difference existed among the three formulas, with formula #3 containing nanoliposome composed of ceramide having a significant greater effect than either formula #1 (placebo) and formula #2 containing dispersed ceramide. The anti-inflammatory effects were 16.8 for formula #1, 23.0 for formula #2, and 40.4% for formula #3 respectively.

**Human patch test**

The human patch test for assessing primary skin irritation potential was performed to evaluate the anti-irritation effect of nanoliposome composed of ceramide against irritant used in cosmetics such as lactic acid. Results were analyzed through mean visual scores at 30 minutes and 24 hours. To evaluate the skin irritation potency of formula #4~#7 manufactured for this patch test (Table 1), scores for the

irritancy were divided into 5 groups; 0~0.99 (Grade I, no irritation), 1.0~1.99 (Grade II, slight irritation), 2.00~2.99 (Grade III, moderate irritation), 3.00~4.99 (Grade IV, strong

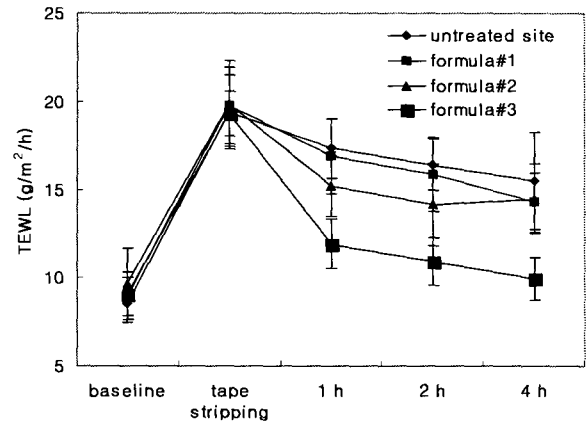


Fig. 1. Time course of TEWL after tape stripping. \*\*, Significantly different at p<0.01 compared to untreated site by the student's t-test

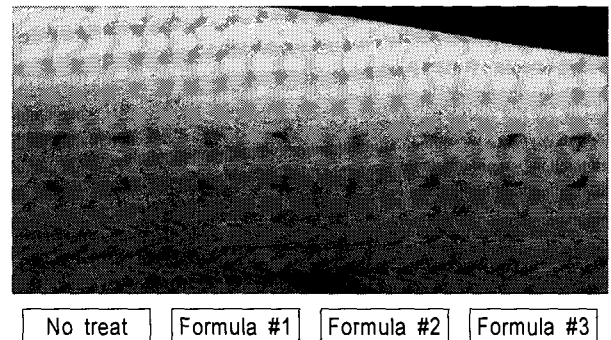


Fig. 2. Erythematous reaction induced by topically applied methyl nicotinate.

Table 2. Inhibitory effect of flush induced by 3 mM methyl nicotinate.

Test materials	Δ a* value	Anti-inflammation effect (%)
No treat	1.61	
Formula #1	1.34	16.8
Formula #2	1.24	23.0
Formula #3	0.96	40.4

Table 3. Results from human patch test with various formulas.

Formulas	Results of evaluation (No. of subjects)								Mean score (n=20)	Assessment	Grade
	30 min				24 hr						
	±	+	++	+++	±	+	++	+++			
#4	5	-	-	-	-	-	-	-	1.04	Slight irri.	II
#5	11	-	-	-	-	-	-	-	2.29	Moderate irri.	III
#6	9	-	-	-	-	-	-	-	1.88	Slight irri.	II
#7	6	-	-	-	-	-	-	-	1.25	Slight irri.	III

irritation) and 5.00 (Grade V, severe irritation). Table 3 shows the results of patch test with various formulas. The mean score of formula #4 (negative control) which did not contain both irritant (lactic acid 5.0%) and anti-irritant was 1.04. As shown in Table 3, the nanoliposome composed of ceramide reduced the mean scores from 2.29 for formula #5 to 1.25 for formula #7. On the whole, the mean score of formula containing nanoliposome composed of ceramide as an anti-irritant showed about 45% reduction as compared to those of positive control.

### Stinging potential test

Stinging potential test for assessing subjective (sensory) irritation potential was performed to evaluate the anti-sting effect of nanoliposome composed of ceramide against lactic acid. Table 4 shows the results of stinging potential test with various formulas. The dispersed ceramide reduced the response rates (%) from 2.33 for formula #5 (lactic acid 5.0%) to 1.93 for formula #6, and nanoliposome composed of ceramide reduced the response rates (%) from 2.33 for formula #5 to 1.74 for formula #7. As can be seen in Table 4, this nanoliposome composed of ceramide was very effective at reducing sting against irritant used in cosmetics, especially lactic acid. Overall, treatment with nanoliposome composed of ceramide as an anti-irritant reduced stinging by about 25%.

### *In vitro* skin penetration test

To evaluate the influence of nanoliposome composed of ceramide on the skin penetration of lactic acid in formulations, we performed *in vitro* skin penetration test using horizontal Franz diffusion cells with skin membrane prepared from hairless mouse. The receptor solution was analyzed, and a plot was made of the total lactic acid penetrating over time. The flux  $J$  was calculated from this diagram as the steady state or linear portion of the flux curve. Flux is generally reported as micrograms per centimeter squared per hour ( $\mu\text{g}/\text{cm}^2/\text{h}$ ). Fig. 3 reports the diffusion kinetics of lactic acid from the manufactured topical formulations. The calculated skin flux for lactic acid, which was incorporated into the different topical forms are reported in Table 5. As shown in Fig. 3 and Table 5, skin flux for lactic acid from formula #3 containing nanoliposome composed of ceramide was much lower than those of formula #1 (placebo) and formula #2 containing dispersed ceramide as a function of time. So, we found that the anti-irritation

Table 4. Results from Stinging potential test with various formulas.

Test materials	Response score	Response rate (%) (n=20)	Anti-sting effect (%)
Formula #5	98	2.33	17.17
Formula #6	81	1.93	
Formula #5	98	2.33	25.32
Formula #7	73	1.74	

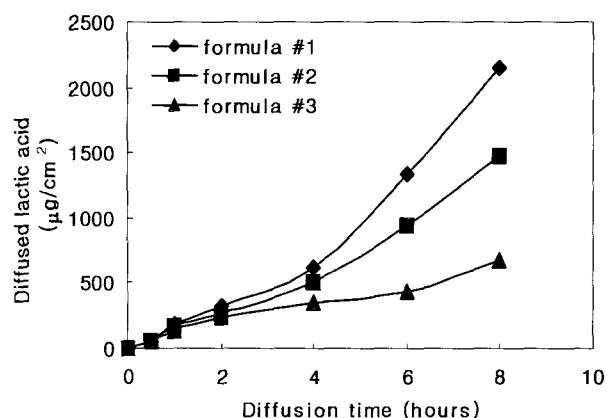


Fig. 3. *In vitro* diffusion kinetics of lactic acid incorporated into the reported topical dosage forms

Table 5. The calculated skin flux for lactic acid from the formulas.

	Skin flux ( $\mu\text{g}/\text{cm}^2/\text{h}$ )		
	Formula #1	Formula #2	Formula #3
Early 0.5 h	173.50	175.34	176.34
Early 4 h	244.14	196.83	138.09
Latter 4 h	596.31	378.98	127.31

effects of nonliposome containing ceramide were due to reduced penetration rate of irritants.

From the results, we could found that a formula containing nanoliposome composed of ceramide had excellent effects on reinforcement of skin barrier function and anti-irritation compared to placebo and formula containing dispersed ceramide. Conclusively, we suggest the potentialities of nanoliposome composed of ceramide as an anti-irritant against various irritants used in cosmetics.

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**초록 : 세라마이드를 구성성분으로 하는 나노리포솜의 응용 - 화장품에서의 자극완화제**

조병기<sup>1</sup> · 안기웅<sup>1</sup> · 신봉수<sup>1</sup> · 정지현<sup>1</sup> · 박해룡 · 황응일<sup>\*</sup>  
 (경남대학교 식품생명공학부, <sup>1</sup>코리아나화장품 연구소)

본 연구는 화장품에서 사용되는 다양한 자극원에 대한 자극완화제로서의 세라마이드를 구성성분으로 하는 나노리포솜의 잠재적 가능성을 알아보고자 하였다. 세라마이드는 인체로부터 수분 손실을 막고, 외부의 물리적, 화학적, 그리고 미생물에 의한 손상으로부터 신체를 보호함으로써 인간의 생리작용에 있어 중요한 부분을 담당하는 것으로 알려진 표피 투과 장벽의 주요한 구조적 구성 성분이다. 본 연구 결과에 의하면 피부 장벽 기능 강화와 자극완화 효과가 제형 내에 단순히 분산된 세라마이드보다 세라마이드를 구성성분으로 하는 나노리포솜을 함유하는 경우 보다 우수하게 나타났다. 그리고, 자극원으로서 제형 내 함유되어 있는 젖산의 피부 투과도에 있어서 세라마이드를 구성성분으로 하는 나노리포솜의 영향을 평가하기 위해 무모 생쥐에서 얻어낸 피부 막으로 horizontal Franz diffusion cells을 이용한 *in vitro* 피부 투과 시험을 수행해 보았다. 시험 결과, 세라마이드로 구성된 나노리포솜의 항자극 효과는 자극원의 피부 투과도를 감소시키는 것으로 확인하였다. 결론적으로, 본 연구에서는 새로운 자극완화 시스템의 개발이 가능하였고 이러한 세라마이드를 구성성분으로 하는 나노리포솜을 화장품에 적용 가능하였다