

Facilitated Skin Permeation of Oregonin by Elastic Liposomal Formulations and Suppression of Atopic Dermatitis in NC/Nga Mice

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To develop an external preparation of oregonin (ORG) for the treatment of atopic dermatitis (AD), conventional creams (CC) and elastic liposomes (EL) containing ORG have been formulated and examined for their *in vitro* skin permeation properties and *in vivo* therapeutic efficacy assessments. EL, consisting of soybean phosphatidylcholine and Tween 80 (85 : 15 w/w %), were of flexible nanocarriers: they were about 130 nm in size and had a 4-fold greater deformability index than conventional liposomes. In a skin permeation study using a Franz diffusion cell mounted with depilated mouse skin, liposomal systems were superior to cream, revealing greater flux values. Both CC and EL were diversified with the addition of Trans-activating transcriptional activator (Tat) peptide, a sort of cell penetrating peptide, and subjected to *in vivo* efficacy evaluations in NC/Nga mice with AD-like lesions. On clinical observation for skin severity, rapid and profound improvement was observed in the treatment group with Tat-added liposomes (EL/T), showing a significant difference ($p < 0.05$) versus Tat-added cream. The results indicated that EL/T treatment is effective for normalizing the immune-related responses and alleviating AD, evaluated as changes in the levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin (IL)-4, immunoglobulin E (IgE), and eosinophils in skin or blood.

Keywords oregonin; elastic liposome; atopic dermatitis; NC/Nga mouse; Trans-activating transcriptional activator peptide

Atopic dermatitis (AD) is a chronic inflammatory skin disease, occurring most commonly during early infancy and childhood. The skin of an AD patient is susceptible to environmental allergens and becomes red, flaky and itchy.¹⁾ Recent progress in pathogenesis has revealed that AD is associated with abnormal immunological responses. Cytokines including interleukin (IL)-4 and IL-5 activate mast cells and eosinophils, and can result in elevated levels of immunoglobulin E (IgE).²⁾ Furthermore, inflammation-related enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are pivotal for provoking the inflammatory response in dermatitis.^{3,4)} Even though corticosteroids have so far been widely used for the treatment of AD, long-term administration of steroidal drugs is very restricted due to severe side effects.⁵⁾ Less toxic natural compounds are being sought as new potential anti-AD agents.⁶⁾

Oregonin (ORG) is a diarylheptanoid derivative, (5*S*)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-beta-D-xylopyranoside, isolated from the bark of *Alnus japonica*.⁷⁾ Various biological activities of ORG including an anti-oxidative effect,⁸⁾ inhibitory effects on nitric oxide synthesis and COX-2 expression,^{9,10)} and an immunomodulatory effect¹¹⁾ have been reported. Recently, it has been further investigated as a natural remedy for the treatment of autoimmune diseases, including AD. On topical applications of ORG, it has to be delivered to the relevant skin layers of the epidermis and dermis. However, the skin permeation of ORG to the deeper dermal layer is very limited because of its hydrophilicity. Although a partially disturbed barrier in AD can favor drug permeation through the skin, an efficient delivery system is still the main concern for the development of topical preparations.¹²⁾

Liposomes have been widely introduced as a non-toxic nanocarrier which can encapsulate both polar and nonpolar

compounds. They usually promote skin permeation of a drug by intact vesicular skin penetration, a penetration enhancing effect, or penetration of liposomes through the transappendageal route.^{13,14)} Elastic liposomes (EL), consisting of phospholipids and an edge activator are one of the novel liquid-state vesicles for topical delivery of drugs. In comparison to conventional liposomes (CL), EL are generally able to respond to an external stress by rapid shape transformation and penetrate the skin efficiently in terms of quantity and depth.^{15,16)} Furthermore, since liposomal vesicles composed of alternating lipid and water phases are structurally similar to the skin constitution, the application of liposomes moisturizes the skin and reduces the irritation resulted from allergic skin disorders.¹⁷⁾ It has been reported that addition of Tat (Trans-activating transcriptional activator) peptide would facilitate the skin penetration and transportation of drug into immune cells.^{18,19)} This short peptide demonstrated the efficiency of translocation of the various molecular cargos with no apparent toxicity.²⁰⁾

In this experiment, ORG-containing external preparations of either cream type or liposomal systems were formulated in the presence or absence of Tat peptide. Skin permeation profiles *in vitro* and delivery enhancing capacity were characterized. Therapeutic improvements of AD in NC/Nga mice were evaluated by clinical skin severity scores, immunohistochemical analyses of iNOS and COX-2 levels in skin, along with alterations of IL-4, IgE and eosinophil levels in blood.

MATERIALS AND METHODS

Animals Male four-week-old albino mice (CD-1[®]) and female five-week-old NC/Nga mice were purchased from Orient Bio (Kyungki-Do, Korea) and Charles River (Yokohama, Japan), respectively. They were kept in specific

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pathogen-free conditions with food and water freely available. All animal experiments were performed in accordance with the NIH guideline "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Chung-Ang University in Seoul, Korea.

Reagents ORG (purity >95% by HPLC) was provided by the Chung-Ang University Pharmacognosy Laboratory (Seoul, Korea). Soybean phosphatidylcholine (PC), cholesterol, Tween 80 and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich Co. (St. Louis, U.S.A.). Tat peptide (Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg, 9mer) was synthesized from Pepttron Co. (Taejun, Korea). Polyglyceryl-3-methylglucose distearate, stearic acid, cetyl alcohol, mineral oil and glycerin used for cream preparation were kindly provided by LG Life Sciences Ltd. (Seoul, Korea). Sodium carboxymethylcellulose (Na-CMC) was purchased from Duksan Pure Chemical Co. (Kyungki-Do, Korea). All other chemicals and reagents were purchased from commercial sources and were of analytical grade. Doubly distilled water was used for all experiments.

Preparation of Cream Formulations A conventional oil-in-water type cream (CC) containing 1.0 w/w % ORG was prepared as follows. Water-soluble ORG was dissolved in aqueous phase consisting of glycerin (10%) and distilled water. The oil phase consisting of mineral oil (7%), stearic acid (5%) and cetyl alcohol (2%) was melted at elevated temperature and polyglyceryl-3-methylglucose distearate (3%) was used as an emulsifier. The oil phase was added to the aqueous phase and homogenized for 10 min, degassed and cooled under vacuum. Tat-added conventional cream (CC/T) was prepared by mixing Tat peptide into CC at the concentration of 0.2 w/w % and re-homogenizing for 3 min. Both cream formulations were then stored for about 48 h before undergoing evaluation.

Preparation of Liposomal Formulations EL were prepared by a reverse phase evaporation (REV) method.^{21,22} Briefly, PC and Tween 80 (85 : 15 w/w %) were dissolved in methanol and chloroform (1 : 1) in a round bottom flask. The aqueous phase containing ORG (50 mg/ml) was then mixed with the organic phase and sonicated in a bath sonicator (Model 2210, Branson Ultrasonics Co., U.S.A.) for 5 min. It was attached immediately to a rotary evaporator (Rotary Evaporator, Super fit, Ambala, India) to dry the contents under vacuum until gels were formed. The gels were then hydrated with distilled water and extruded ten times through a 200 nm polycarbonate membrane filter (Millipore, U.S.A.). Similarly, CL were prepared using PC and cholesterol (85 : 15 w/w %). The final lipid concentration in all vesicular formulations was 30 mg/ml and the drug concentration was 1%. Tat-added elastic liposomes (EL/T) were formulated by adding Tat peptide to EL in the concentration of 0.2 (w/w %). A thickening agent, Na-CMC, was added to all liposomal formulations at the concentration of 1.5 (w/v %) to improve the application of formulations to the dorsal skin of the mice.

Characterization of Liposomal Formulations Size and Zeta Potential: Liposomes were diluted with an appropriate volume of water and examined for size distribution and zeta potential using a dynamic light scattering particle size analyzer (Zetasizer Nano-ZS, Malvern Instrument, Worcestershire, U.K.) with a 50 mV laser at a scattering angle of 90 °C.

All measurements were carried out under ambient conditions and in triplicate.

Loading Amount and Loading Efficiency: Unencapsulated drug was separated from liposomes using an ultrafiltration-stirred cell (Millipore, U.S.A.).²³ Liposomal suspensions were held over the filtration membrane (pore size 30 kDa) in the pressurized cell (20 psi). The filtrate and supernatant were analyzed for ORG by HPLC. The loading amount per total lipid was calculated by $[(T-C)/W] \times 100$ and the entrapment efficiency of the liposomes was calculated by $[(T-C)/T] \times 100$, where T is the total amount of drug initially added, C is the amount of drug detected in the supernatant, and W is the amount of lipid used in the formulations.²⁴

Elasticity of Vesicle Membrane: The vesicle suspension was driven through a microporous filter by an external pressure of 2.5 bars.²⁵ The size of the vesicles was monitored by dynamic light scattering measurement before and after the filtration. The elasticity of vesicle membranes was then calculated by using the following formula as reported by van den Bergh *et al.*²⁶: $D = J \times (r_v/r_p)^2$, where D is the deformability index of the vesicle membrane, J is the amount of vesicle suspension extruded for 5 min, r_v is the size of vesicles after extrusion, and r_p is the pore size of the barrier.

In Vitro Skin Permeation Study *In vitro* permeation study was conducted with vertical Franz diffusion cells as described previously.²⁷ Skin tissues were obtained from hairy albino mice. The dorsal hair of mouse skin was carefully removed using electric clippers and rinsed with phosphate buffer. A circular piece of dorsal skin was then carefully mounted onto the receiver compartment of the diffusion cells with the stratum corneum facing in the direction of the donor compartment. The receptor chamber was filled with 10 mM PBS (pH 7.4) solution and maintained at 32 °C. Each formulation containing the equivalent amount of ORG (5.0 mg) was applied to the skin surface, which had an available diffusion area of 1.76 cm². The aliquots (0.5 ml) were withdrawn at predetermined time intervals and analyzed by HPLC. The cumulative amount of drug permeated per unit area was plotted as a function of time and the steady-state permeation rate (J_{ss}) was calculated from the slope.

HPLC Assay of ORG The quantitative determination of ORG was performed by HPLC using acetonitrile–water (20 : 80) as a mobile phase at a flow rate of 1 ml/min. The HPLC system consisted of a pump (L-2130), UV detector (L-2400), a data station (LaChrom Elite, Hitachi, Japan), and a 15 cm C₁₈ column (Shiseido, Tokyo, Japan). The column eluant was monitored at 262 nm, and the ORG peak was separated with a retention time of 7.5 min.

Induction of AD in Mice and Topical Application of ORG AD-like lesions were induced by the topical application of diphenylcyclopropanone (DPCP), a well-known contact allergen, to NC/Nga mice as described earlier.^{28,29} The hair of mouse skin was carefully removed using electric clippers and stimulated for 10 weeks by 0.1% DPCP solution under ambient conditions. AD was characterized by itching, erythema and hemorrhage, followed by edematous superficial erosion, deep excoriation, scaling, and dryness of the skin.³⁰ For topical application, regrown hairs on the back were removed a day before the experiment. The mice were treated daily for four weeks with drug-free CC base alone (control) or drug-containing topical formulations of CC/T,

EL, and EL/T.

Clinical Observation of Skin Severity The clinical severity of the dermatitis in NC/Nga mice was observed for four weeks and scored as previously described.²⁹⁾ A total score for skin severity was defined as the sum of individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each sign and symptom, such as erythema, edema, oozing or crust, excoriation, lichenification, and dryness.

Measurement of iNOS and COX-2 Levels in Skin Mice were sacrificed at four weeks after ORG treatment. An area of approximately $1.5 \times 1.5 \text{ cm}^2$ was excised, and the subcutaneous fat and blood vessels were carefully dissected away. Expression levels of iNOS and COX-2 in skin were measured by a method previously described.³¹⁾ For immunohistochemical examination, skin biopsies were frozen and 5- μm -thick sections were cut. Sections were deparaffinized in xylene, rehydrated in ethanol solution, and treated with hydrogen peroxide (0.3%) to quench endogenous peroxidase activity. For the detection of iNOS and COX-2, slides were incubated with rabbit polyclonal anti-iNOS (1:100 dilution; BD Transduction Laboratories) or rabbit polyclonal anti-COX-2 (1:50 dilution; Santa Cruz Biotechnology). They were then incubated with biotinylated secondary antibody (1:500, goat anti-rat and goat anti-rabbit, Amersham, U.S.A.) followed by horseradish peroxidase–streptavidin conjugates and 3,3'-diaminobenzidine (Sigma-Aldrich). Later, the slides were counterstained with hematoxylin and examined by light microscopy. For Western blotting, skin tissues were lysed in a buffer containing 50 mM Tris–Cl, 150 mM NaCl, 0.02% sodium azide, 100 $\mu\text{g}/\text{ml}$ phenylmethanesulfonyl fluoride, 1% Triton X100 and 1 $\mu\text{g}/\text{ml}$ aprotinin. After centrifugation, the supernatant was run on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For iNOS and COX-2 detection, the electrophoretically resolved proteins were transferred onto nitrocellulose membranes (Osmonics, Milwaukee, WI, U.S.A.). The membrane was incubated with anti-iNOS and COX-2 polyclonal antibody (1:1000 in 5% BSA, Chemicon, CA, U.S.A.) and then washed three times with Triton X100 containing PBS solution. The proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, U.K.).

Measurement of IL-4, IgE and Eosinophil Levels in Blood The amount of IL-4, IgE and eosinophil in blood was quantified according to methods previously described.^{32,33)} Blood was collected from the retro-orbital plexus of mice under anesthesia by ether. Serum was obtained by centrifugation at 12000 rpm and stored at -20°C until use. The level of IL-4 was measured using a mouse IL-4 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, U.S.A.). The amount of total IgE in the serum was determined using a mouse IgE ELISA kit. Eosinophil count was conducted on 30 μl of capillary blood diluted six-fold with saline. Microscopic differential counts were performed on Wright–Giemsa stained blood smears. Eosinophil counts were calculated from differential counts.

Statistical Analysis All data are expressed as the mean $\pm$ S.D. Statistical significance was confirmed by Student's *t*-test and considered to be significant at $p < 0.05$, unless otherwise indicated.

RESULTS AND DISCUSSION

Characteristics of Topical Cream and Liposomal Formulations All cream formulations were prepared successfully and appeared as white, opaque and homogeneous semi-fluids with no bleeding or phase separation. The drug contents in the formulations were over 98%. The addition of Tat peptide did not have any influence on the physical characteristics or drug stability of the cream formulation.

The characteristics of liposomal formulations were evaluated by vesicular size, polydispersity index (PDI), zeta potential, loading amount, efficiency and deformability index (Table 1). Except for the deformability, other parameters were in a similar range for both CL and EL. The vesicular size was measured to be approximately 130 to 140 nm on average, which is considered an ideal size for skin delivery. In an earlier study,³⁴⁾ EL with a size of 120 nm showed a maximum effect with respect to both accumulation of hydrophilic fluorescent compound in the deeper skin layers and recovery in the receptor compartment of the Franz diffusion cell: as compared to larger ones of 200 and 370 nm, about 4.5- and 7.0-fold greater accumulation and about 1.5- and 2.5-fold greater recovery, respectively. A low polydispersity index of less than 0.3 indicates a narrow and homogeneous size distribution. The liposomal vesicles elicited a nearly neutral surface charge with a slight negative value contributed by deprotonated PC.

The deformability of EL, a crucial feature of EL formulations, was about 4-fold greater than that of CL. The incorporation of an edge activator, Tween 80, destabilized lipid bilayers, thereby increasing the deformability of vesicles.³⁵⁾ Various edge activators including Tween 80, Span 80, sodium cholate, sodium deoxycholate, and glycyrrhizin have been employed to prepare deformable liposomes.^{35–37)} Deformability was found to be maximized when the surfactant weight ratio is about 15%, generally providing a 3–10 times higher deformability index compared to conventional vesicles. In other words, stress-dependent adaptability expressed as deformability index offers a unique property to pass through the skin barrier with ease, which in turn provides an adjuvant effect for ORG permeation. The addition of Tat peptide resulted in no changes in the physical characteristics of the EL formulation.

In Vitro Skin Permeation of ORG The *in vitro* permeation profiles of ORG in various topical formulations including CC, CC/T, CL, EL and EL/T are shown in Fig. 1. There

Table 1. Characteristics of ORG-Loaded Elastic and Conventional Liposomal Formulations

	CL	EL
Composition (w/w)	PC : Cholesterol (85 : 15)	PC : Tween 80 (85 : 15)
Size distribution (nm)	140.2 $\pm$ 4.6	130.6 $\pm$ 3.4
PDI	0.142	0.132
Zeta potential (mV)	−3.6 $\pm$ 1.4	−9.3 $\pm$ 2.6
Loading amount (mg/lipid) ^{a)}	0.18 $\pm$ 0.05	0.17 $\pm$ 0.07
Loading efficiency (%)	58.1 $\pm$ 5.4	55.2 $\pm$ 6.2
Deformability	15.5 $\pm$ 3.9	60.2 $\pm$ 5.1

Abbreviations are as follows: CL, conventional liposomes; EL, elastic liposomes; PC, phosphatidylcholine; PDI, polydispersity index. ^{a)} mg/lipid indicates the amount of drug loaded per total lipid used. Values represent mean $\pm$ S.D. ($n=3$).

was no distinct lag time for skin permeation and the cumulative amount that permeated increased linearly for the entire period of the study, indicating that permeation steady-states were reached rapidly. The order of ORG penetration at 24 h was as follows: EL/T > EL > CC/T > CL > CC. However, if we look further into the details of the pattern, slight differences in the slopes could be recognized. In earlier permeation (before 8 h), there was little difference in slopes between the various formulations. In later permeation (after 8 h), on the contrary, the slopes became steeper, especially for liposomal systems, showing the greatest flux value in EL/T. These phenomena could be extended to the explanation that liposomal systems are superior to the cream formulation, and further, elasticity rendering and Tat peptide addition to liposomes are beneficial for the dermal delivery of ORG.

The values of steady-state flux ($\mu\text{g}/\text{cm}^2/\text{h}$) obtained were in the range of 8 to 14 for different formulations (Table 2). All liposome formulations yielded greater values compared to creams. Tat peptide addition to CC or EL increased the flux by about 20% in both cases. The percentage of permeation was calculated from the cumulative amount permeated in the receptor phase at 24 h divided by the amount of drug loaded in the samples. Although liposomal systems showed greater permeation, the amount permeated for every formulation was in the range of about 6 to 10%. For further comparison, enhancement ratio (ER) was expressed as a relative ratio of the flux values. In accordance with the permeation pattern, the advantage of liposomal formulations was obvious. EL/T formulation brought a great increment: about 20% against EL, 40% against CL, and 80% against CC.

Oil-in-water type cream is conventionally used in cosmetics or pharmaceuticals with non-sticky, non-oily and water-washable properties. Application of cream to the surface of skin would increase the hydration of the stratum corneum, and improve the permeation of drug into the skin. However, most cream formulations do not penetrate deeply into the

skin, but rather remain confined to upper layers of the stratum corneum.¹²⁾ Moreover, since ORG is distributed in the external phase in cream due to its water solubility, it is perhaps difficult to expect a penetration enhancing effect of the inner oil phase.

In contrast, the lipid vesicular systems for improving skin delivery of hydrophilic drug have been well demonstrated. Several studies have proposed possible mechanisms by which vesicles could improve skin delivery of drugs.^{38,39)} First, intact vesicles carrying a drug enter the skin directly through both the stratum corneum and the follicular shunt. Secondly, the vesicles act as a penetration enhancer, leading to modification of the arrangement of intercellular lipid lamellae. It has been proved that elastic vesicles are more effective than conventional vesicles in the enhancement of drug delivery across animal and human skin. EL rapidly entered the deeper layers of the stratum corneum and could reach layers almost as deep as the stratum corneum-viable epidermal junction within 1 h.⁴⁰⁾ The higher stress-dependent adaptability of EL compared to CL enabled them to squeeze between the cells in the stratum corneum, which allows them to trespass the intact skin spontaneously under the influence of the naturally occurring transcutaneous hydration gradient.

On the other hand, the addition of Tat peptide to EL improved the skin delivery of ORG by up to 20%. This result is consistent with the earlier reports, describing that covalent or non-covalent combination with cell penetrating peptides (CPPs) including Tat peptide exhibited powerful delivery of drug to the epidermis, dermis, panniculus adiposus, and even hypodermis.¹⁹⁾ Both the peptide and the peptide-drug conjugate seemed to enter a layer of cells *via* a macropinocytosis mechanism, saturate it, and enter the adjacent layer forming a gradient from the outer to the inner layers of cells. This process facilitates the skin delivery of drug.^{18,19)} Delivery of cyclosporine A to immune cells including dermal T cells was also enhanced by the addition of an arginine heptamer, a kind of CPP, in which significant reduction in inflammation was observed in a mouse model of contact dermatitis.¹⁸⁾

In Vivo Therapeutic Efficacy for Skin Severity
NC/Nga mice with AD-like lesions manifested severe pruritis, dryness and eczema that showed no significant differences in the severity levels between mice. Topical application of ORG reduced considerably these symptoms in all treated groups, showing a complete recovery within four weeks after the treatment (Fig. 2). However, the control group revealed an incomplete recovery even after four weeks. Evaluation of SCORAD for skin severity as shown in Fig. 3 revealed both elastic liposomal formulations started to show significantly different improvements within a week, which continues to reduce gradually the values up to four weeks. However, in the CC/T-treated group, a significant difference was observed

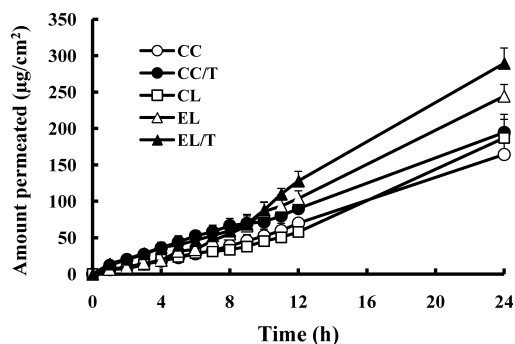


Fig. 1. Permeation Profiles of ORG through Depilated Mouse Skin for 24 h in Various Formulations

Values represent mean $\pm$ S.E. ($n=4$).

Table 2. Permeation Parameters of ORG through Depilated Mouse Skin in Various Formulations

	CC	CC/T	CL	EL	EL/T
J_{ss} ($\mu\text{g}/\text{h}/\text{cm}^2$) ^{a)}	7.96 ± 0.92	9.79 ± 1.22	9.92 ± 0.97	11.71 ± 0.93	14.27 ± 1.57
Permeated (%) ^{b)}	5.78 ± 0.76	6.85 ± 0.63	6.58 ± 0.57	8.60 ± 0.22	10.19 ± 0.56
ER ^{c)}	1.00	1.23	1.25	1.47	1.79

Abbreviations for formulations: CC, conventional cream; CC/T, Tat-added CC; CL, conventional liposomes; EL, elastic liposomes; EL/T, Tat-added EL. a) Flux obtained from the slope of the permeation profile in Fig. 1. b) Calculated from the cumulative amount permeated at 24 h divided by the amount of drug loaded in the samples. c) Enhancement ratio, relative to CC. Values represent mean $\pm$ S.D. ($n=4$).

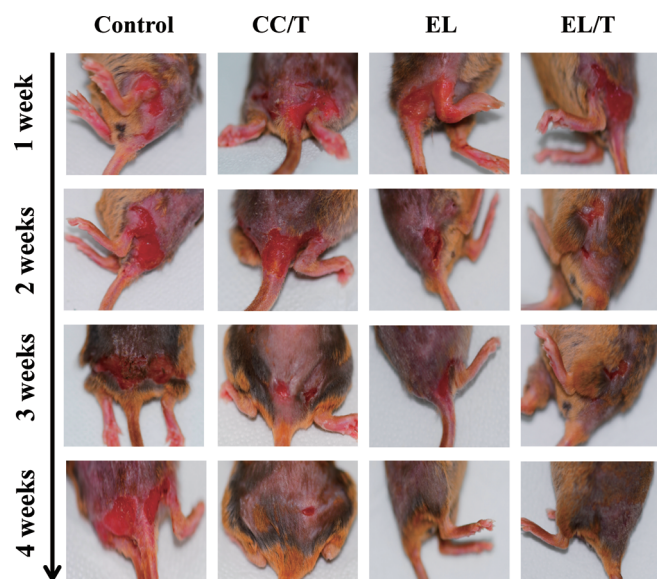


Fig. 2. Clinical Observation for AD-Like Skin Lesions in NC/Nga Mice after Topical Administration of Various Formulations; Drug-Free Cream Base (Control), Tat-Added Conventional Cream (CC/T), Elastic Liposomes (EL) and Tat-Added Elastic Liposomes (EL/T)

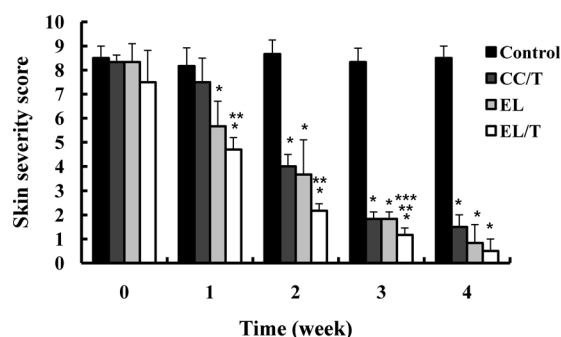


Fig. 3. Changes in Skin Severity Score after Topical Application of Various Formulations; Control, CC/T, EL and EL/T

A total score for skin severity was defined as the sum of individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each sign and symptom, such as erythema, edema, oozing or crust, excoriation, lichenification, and dryness. Bar represents S.D. ($n=3$) and the statistical analysis was performed using the Student's *t*-test (* $p<0.05$ versus control; ** $p<0.05$ versus Tat/CC; *** $p<0.05$ versus EL).

from two weeks after the treatment. In particular, the EL/T-treated group resulted in the greatest reduction in values, showing significant differences from the CC/T-treated group at a week and from the EL-treated group at three weeks. Although the improvement was observed in all three treatment groups, more rapid and profound improvement was found in those groups with elastic liposomal formulations, especially for EL/T formulation. This result correlates very well with *in vitro* skin permeation outcomes.

Suppression of iNOS and COX-2 Levels There are several reports demonstrating that ORG is a potent inhibitor of iNOS and COX-2: *e.g.*, inhibition of NO synthesis in a dose-dependent manner through the suppression of iNOS mRNA expression and inhibitory effect on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced COX-2 expression in human breast epithelial cells.^{9,10} As shown in Fig. 4, in the control group, immunohistochemical observation revealed the abundant expression of iNOS as well as COX-2 in dermis or epidermis of NC/Nga mice bearing AD symptoms. A brown spot is an indication of positive staining for the iNOS or COX-2 enzyme. Upon treating AD skin lesions with ORG, considerable reduction in the number of brown spots in epidermis and dermis was observed in all three treatment groups. In particular, brown spots had almost totally faded away in the EL- or EL/T-treated groups, which is different from the CC/T-treated group. In accordance with the clinical observation on skin severity, all three treatment groups resulted in great declines in protein expression levels, giving better improvement in those groups with elastic liposomal formulations (Fig. 5). Although more than 50% reduction was obtained by CC/T treatment, both elastic liposomal formulations decreased even further to about 30%. Also, the EL/T-treated group exhibited the best result, showing significant differences from the CC/T-treated group for iNOS reduction and from the CC/T- or EL-treated group for COX-2 reduction.

Suppression of IL-4, IgE and Eosinophil Levels Several studies reported the elevation of immune responses including IL-4, IgE and eosinophil levels in NC/Nga mice with AD-like lesions.^{2,41–43} It is well established that the elevation of serum IgE in AD may be due to the Th1/Th2 imbalance.

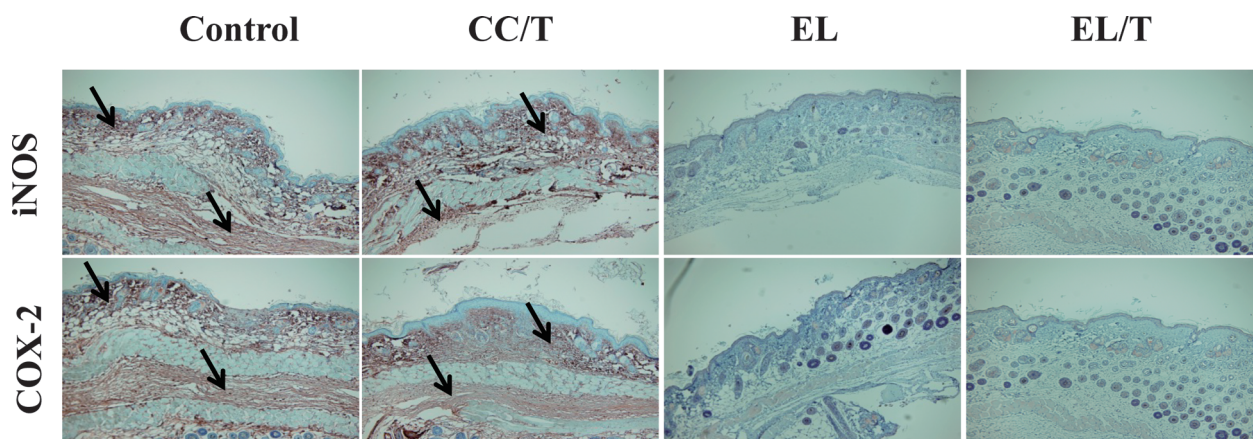


Fig. 4. Immunohistochemical Analyses of iNOS and COX-2 Levels in NC/Nga Mouse Skin after Topical Application of Various Formulations; Control, CC/T, EL and EL/T

Skin sections were incubated with primary iNOS antibody or COX-2 antibody, which in turn incubated with biotinylated secondary antibodies, horseradish peroxidase-streptavidin conjugates and 3,3'-diaminobenzidine, then counterstained with hematoxylin for light microscopy. A brown spot (arrow) indicates positive staining for the iNOS or COX-2 enzyme. Original magnification $\times 400$.

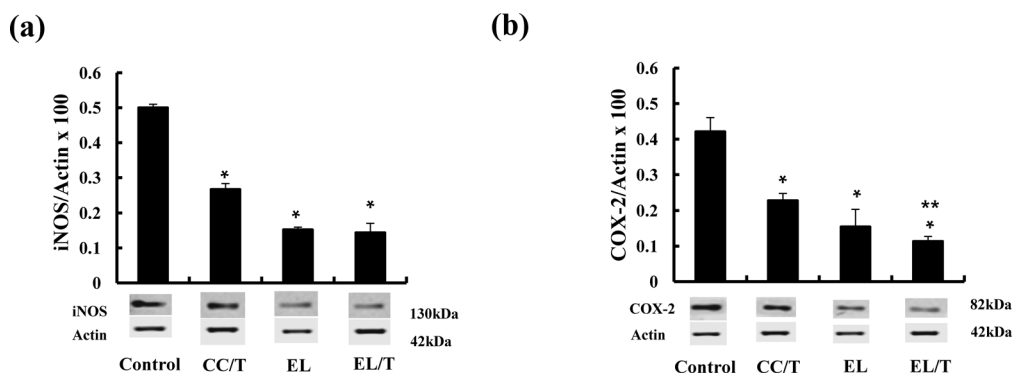


Fig. 5. Alterations of (a) iNOS and (b) COX-2 Levels in NC/Nga Mouse Skin after Topical Application of Various Formulations; Control, CC/T, EL and EL/T

The levels were determined by Western blotting. Bar represents S.D. ($n=3$) and the statistical analysis was performed using the Student's *t*-test (* $p<0.05$ versus control; ** $p<0.05$ versus CC/T).

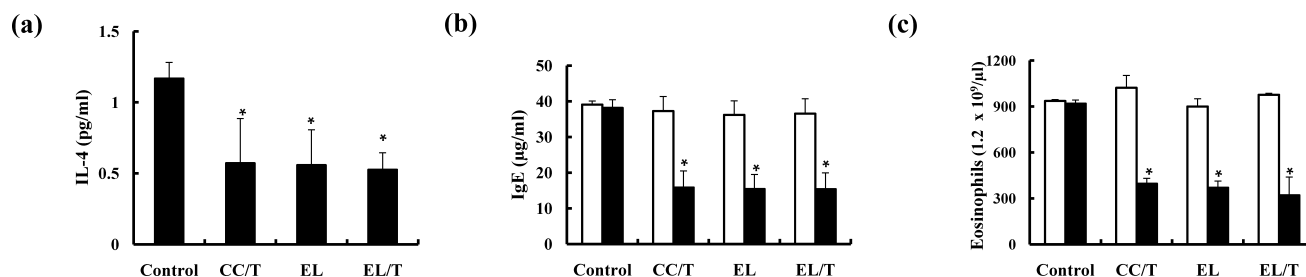


Fig. 6. Alterations of IL-4, IgE and Eosinophil Levels in NC/Nga Mouse Blood after Topical Application of Various Formulations; Control, CC/T, EL and EL/T

Bar graphs represent for both before the treatment (empty) and four weeks after the treatment (filled). (a) The serum IL-4 levels were measured using ELISA kit. (b) The serum IgE levels were measured using ELISA kit. (c) Blood eosinophil number was counted by the microscopic differential counts. Bar represents S.D. ($n=3$) and the statistical analysis was performed using the Student's *t*-test (* $p<0.05$ versus control).

ance skewed to Th2, in which Th2 cytokines like IL-4 and IL-13 play a key role for the hyperproduction of IgE.⁴⁴⁾ Furthermore, eosinophils generate some kinds of leukotrienes, which amplify the inflammatory cascades by acting as a chemotactic factor or by triggering the release of cytotoxic protein.⁴⁵⁾ In the present study, repeated treatment of DPCP in NC/Nga mice triggered the IgE hyperproduction with up-regulation of IL-4. In the control group, as shown in Fig. 6, the levels were greatly elevated and little changes were observed even after four weeks. But topical application of ORG-containing formulations of CC/T, EL, and EL/T led to remarkable reductions in IL-4 levels to less than a half (Fig. 6a). Consequently, profound decreases in serum IgE levels were observed (Fig. 6b). This trend was very similar to the result for eosinophils, which play a crucial role in aggravating AD symptoms (Fig. 6c). Eosinophil count was normalized to the level of 400 ($1.2 \times 10^9/\mu\text{l}$), which is less than half that of the control group, by the application of three topical formulations.

However, suppression levels for IL-4, IgE and eosinophils revealed the same degree in all treated groups. This might prove the fact that ORG is effective for AD treatment, but the formulation effect exists no longer than four weeks after topical application. The insignificant difference between cream and elastic liposomal formulations could be due to the experimental schedule. After four weeks of drug treatment, the expression levels of the parameters were mostly normalized. If the measurements were carried out at three weeks, the excellence of EL/T could be found. This supposition could be strongly supported by the evidence that pathophysiological

features, as shown in Fig. 3, were noticeably normalized in all groups at four weeks after the treatment in clinical observation. Based on these observations, it is suggested that the AD-associated parameters are quickly normalized in NC/Nga mice by the application of ORG-containing elastic liposomal formulations.

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