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In vivo Skin Deposition and Photoprotection Effect of Genistein in Liposomal Gel Formulations

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ABSTRACT – To enhance the skin delivery of genistein (GT), a soybean isoflavone having antioxidative activity, comparative formulation studies including liposomes were carried out. GT-loaded conventional and elastic liposomal gel showed the enhanced skin deposition and photoprotection effect as well, in comparison to GT suspension. Elastic liposomes composed of soybean phosphatidylcholine and sodium deoxycholate (85:15, w/w%) were superior to conventional liposomes and were of characteristics as follows: about 130 nm in size; 85% encapsulation efficiency of GT; 5.8% skin deposition of applied dose; 40% inhibition effect on UVB-induced H_2O_2 production. Photoprotection effect was closely related to skin deposition of GT. In conclusion, it is possible to suggest that elastic liposomes could be a promising nanocarrier system for efficient skin delivery.

Key words – Genistein, Skin delivery, Elastic liposome, Photoprotection, Antioxidative activity

Genistein (GT), one of the major isoflavones found in Leguminosae, is supplied as dietary to take advantage of various beneficial effects on health. It has been attracted to medical scientific community since epidemiologic studies showed that consumption of soybean-containing diets was associated with a lower incidence of certain human cancers in Asian populations.[1] Further studies revealed that such chemopreventive and antineoplastic effects of GT were related with the antioxidative and inhibiting activities on cell proliferation and angiogenesis.[2]

Moreover, it has been demonstrated that GT has potent antiphotocarcinogenic and antiphotoaging effects. GT significantly inhibited ultraviolet-B (UVB)-induced oxidative DNA damages and production of reactive oxygen species in animal models.[3] GT has been expected to be an effective preventive and therapeutic agent against photocarcinogenesis and photoaging of skin.[4] In practice, those pharmacodynamic activities were greater when GT was topically applied than orally administered to hairless mouse *in vivo* because of poor oral bioavailability.[3]

Skin delivery of GT could be considered as a primary choice. But the stratum corneum acts as a principal barrier for topical or transdermal drug delivery, penetration enhancements are the most concern for the development of optimized formulation. Recently, among the various approaches including nanocarriers, applications of elastic vesicles classified as either phospholipids-based or surfactant-based type were broadly reviewed.[5] Elastic vesicles were more efficient at delivering a drug molecule to the skin in terms of quantity and depth.

Elastic liposomes (EL) have been introduced in the early 1990s as one of the novel liquid-state vesicles for topical delivery of drug,[6,7] since they prolonged the release and demonstrated better biological activity in comparing with conventional liposomes (CL) and ointment. EL consisted of phospholipids and edge activators which could increase the deformability of the bilayer. They are able to respond to an external stress by rapid shape transformation and could be squeezed out through the skin more efficiently.[8] However, even though the permeation enhancing mechanisms of EL are relatively well investigated,[9] skin permeation and deposition degree *in vivo* are not still examined in detail, particularly along with the pharmacodynamic activities of therapeutic agent.

Therefore, in this study, elastic liposomal system was employed to enhance skin delivery of GT and maximize its antioxidative activity. The liposomes were characterized based on size, encapsulation efficiency and drug entrapment, and formulated into pharmaceutical gels. After external applications of gels to hairless mouse *in vivo*, the effectiveness of elastic nanocarrier system was evaluated in terms of skin deposition and antioxidative photoprotection effect.

Experimental

Materials

GT was provided from Rexgene Biotech Co., Ltd. (Seoul, Korea). Soybean phosphatidylcholine, sodium deoxycholate,

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ammonium formate and xylenol orange were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *D*-Sorbitol was purchased from Duksan Pure Chemical Company (Kyungkido, Korea). Ammonium ferrous sulfate was purchased from Junsei Chemical Co. (Tokyo, Japan). Pluronic F127® (poloxamer 407) was purchased from BASF laboratories (Wyandoote, USA). All other chemicals and reagents were purchased from commercial sources and were of analytical grade. Doubly distilled water was used for all experiments.

Preparation and characterization of liposomes

EL and CL were prepared by conventional rotary evaporation and extrusion method.[10,11] Composition of EL was soybean phosphatidylcholine and sodium deoxycholate (85 : 15, w/w%). Sodium deoxycholate was used as an edge activator for giving deformability. CL were made with soybean phosphatidylcholine only.

Briefly, GT, phospholipid and sodium deoxycholate were dissolved in methanol and chloroform (1:1) in round bottom flask. The solvent was later removed by a rotary vacuum evaporator above the lipid transition temperature (Rotary Evaporator, Super fit, Ambala, India) and solvent traces were totally removed by maintaining the lipid film under vacuum overnight. The lipid films were then hydrated with distilled water and extruded ten times through a 100 nm polycarbonate membrane filter (Millipore, USA).

After extrusion procedure of liposomes, the product was ultracentrifuged at 48,000 *g* at 4°C for 30 min in order to separate the liposome-encapsulated drug from the free drug. The supernatant and precipitate were analyzed for GT by HPLC to measure encapsulation percentage. The encapsulation efficiency of liposomes was calculated as follows: $[T-C]/T \times 100$, where T is the total amount of drug that is detected both in the supernatant and sediment, and C is the amount of drug detected only in the supernatant. The final lipid concentration in all vesicular formulations was 30 mM.

Physical characteristics of liposomes such as size distribution and polydispersity index were determined by dynamic light scattering (DLS) method with Zetasizer Nano-ZS (Malvern Instrument, Worcestershire, UK).

Preparation of liposomal gel formulations

Liposomal system was mixed with polymeric gelling agent to increase the viscosity and improve the application of formulation to dorsal skin of the animal. Liposomal gel formulations were prepared by mixing 40% (w/v) poloxamer 407 gel base with appropriate liposome suspensions (1:1, v/v) at 4°C.

HPLC assay of GT

The concentration of GT was determined by HPLC. The HPLC system consisted of a pump (L-2130), UV detector (L-2400, $\lambda = 262$ nm), a data station (LaChrom Elite, Hitachi, Japan) and a 15 cm C_{18} column (Shiseido, Tokyo, Japan). The mobile phase comprised acetonitrile: 50 mmol ammonium formate buffer solution (4 : 6, v/v) and was delivered at a flow rate of 1 mL/min. The injection volume was 50 μL and the relative retention time was found to be 4.0 - 4.2 min.

***In vivo* skin deposition study**

SKH1 hairless mice (6 weeks old, female) were purchased from Orient Bio. Inc. (Kyungkido, Korea) and randomly designed to three groups. Gel formulations of GT suspension (GT-SUS), GT-loaded conventional liposomes (GT-CL) and GT-loaded elastic liposomes (GT-EL) were applied in the same to the dorsal skins of hairless mice by micropipette. After 3 hr, the hairless mice were sacrificed and the dorsal skins were removed and washed five times with phosphate buffer to remove excess drug from the skin surface. And then, the dorsal skins were cut into small pieces and homogenized with 5 mL methanol for 5 min. After centrifugation, amount of GT deposited to the skin was determined by HPLC. Each experiment was repeated in triplicate.

***In vivo* antioxidative photoprotection study**

Hairless mice were randomly designed to different groups with four mice in each group. Gel formulations of GT-SUS, GT-CL and GT-EL containing equivalent amount of active ingredient were applied to the skin 1 hr prior to UVB irradiation. Then mice were exposed to UVB (306 nm) of 15 KJ/m^2 for irradiation using a phototherapy unit with Westinghouse FS40 Sunlamps. The distance between the UV lamps and the dorsal skin of mice was approximately 40 cm. Treatment and irradiation were repeated 3 times weekly. Mice were sacrificed 1 hr after the last UV exposure, dorsal skins were excised. The H_2O_2 determination method described elsewhere[12] was used in the present study with a mild modification. Briefly, skin punches with a diameter of 1 cm from mice were removed and homogenized in 2 mL of balanced salt solution (pH 7.4). The homogenate was filtered and mixed with 10 mL of 40% trichloroacetic acid and centrifuged at 5,000 rpm for 5 min. One hundred micro-liter of the supernatant were than mixed with 900 μL of reaction solution containing 250 μM ammonium ferrous sulfate, 100 μM xylenol orange and 100 μM sorbitol in 25 mM sulfuric acid. Then, it was placed at the room temperature for 30 min and the absorbance of each sample was determined by a spectrometer at 540 nm and concentration of

Table I–*Characteristics of Liposomes*

Parameters	Conventional liposomes	Elastic liposomes
Mean vesicular size (nm)	130.5 ± 24.1	130.6 ± 8.6
Polydispersity index	0.17 ± 0.02	0.17 ± 0.01
Encapsulation efficiency (%)	83.3 ± 4.2	85.1 ± 3.8
Concentration of GT (mg/mL)	1.64 ± 0.21	1.65 ± 0.28

* Data are expressed as mean ± S.D. (n=3).

hydrogen peroxide was calculated.

Statistical analysis

All reported data are mean ± S.D. Statistical significance was checked by Student's t-test and considered to be granted at $P<0.05$, unless otherwise indicated.

Results and Discussion

Characteristics of liposomal formulations

GT-loaded EL and CL were evaluated by vesicular size, polydispersity index, encapsulation efficiency and drug concentration (Table I). The vesicular size of both liposomes was identical, measured as approximately 130 nm in average, revealing no significant differences. It was reported that liposomes with a size of 120 nm diameter showed statistically enhanced penetration into the skin as compared to larger ones.[13] And low polydispersity index less than 0.3 for both EL and CL indicates a narrow and homogeneous size distribution and proves normal preparation. Therefore, these liposomal nanocarriers might be suitable for efficient skin delivery of GT.

Both EL and CL showed the high encapsulation efficiency (approximately 85%) and the similar concentration of GT. It is considered that the high incorporation of GT in lipid bilayer may be attributed to relative lipophilicity of GT having log P value of 1.74 as reported elsewhere.[14] And the supplement of relatively great lipid amount in liposome preparation procedure might attribute to comparatively high encapsulation efficiency of drug.[15]

***In vivo* skin deposition behavior**

Determination of GT amount accumulated in the skin *in vivo* should be investigated to monitor the delivering efficiency of the nanocarriers in reality, since antiphotocarcinogenic and antiphotoaging effects of GT are vigorously taken place in relevant skin layers.[16] The percentile amount of GT deposited in the hairless mouse skin from each formulation is depicted in Figure 1. In comparison to GT suspension (3.3%), both liposomal systems showed enhanced deposition. And EL (5.8%) exhibited a greater value than that of CL (5.0%).

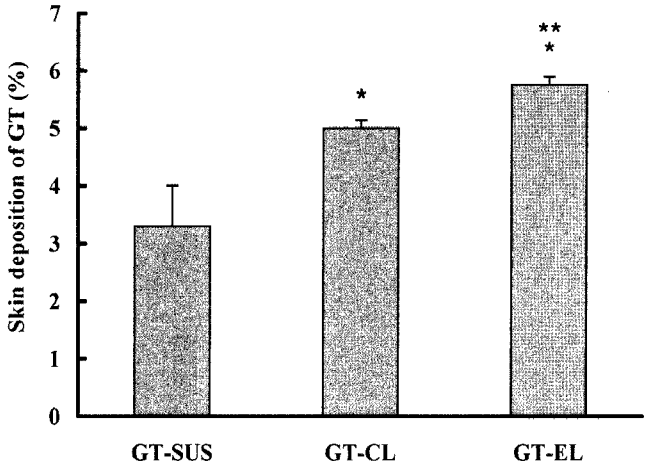

Figure 1–Amount of GT deposited in the hairless mouse dorsal skin from the formulations. The bar represents S.D. and statistical analysis was performed using the Student's t-test (*$P<0.05$ versus GT-SUS; **$P<0.05$ versus GT-CL).

The enhancement in liposomal formulations might support the hypothesis of dual routes for skin penetration of the liposome vesicles.[17] Although it is generally believed that intact liposomes do not penetrate into the compact layers of the stratum corneum, the specific mechanisms of liposome action in the skin strata remain to be enlightened. And skin appendages such as hair follicles, pilosebaceous and eccrine glands may offer an alternative pathway for a diffusing molecule.

Particularly, the use of EL as a vesicular drug carrier could overcome the limitation of low penetration ability of CL or other dosage forms across the skin. A number of reports have demonstrated that elastic vesicles may enhance drug transport by virtue of two functions: as a carrier system as well as a penetration enhancer.[5,18,19] It is believed that penetration capability of elastic carriers is directly related to the properties of both the deformability of the vesicle bilayer and the existence of an osmotic gradient across the skin.

***In vivo* photoprotection effect**

GT inhibits photocarcinogenesis which is related with UVB-induced oxidative events, including hydrogen peroxide production and lipid peroxidation *in vivo*. UVB is well known to generate DNA damage through oxidative stress by increasing levels of reactive oxygen species (ROS),[20] which includes free radicals such as H_2O_2, O_2 and OH•. Earlier studies reported that GT could inhibit the 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-mediated H_2O_2 formation and inflammatory responses in cell culture.[21,22]

Since subacute exposure of hairless mouse skin to UVB substantially increases the level of H_2O_2, pre-treatment of animals

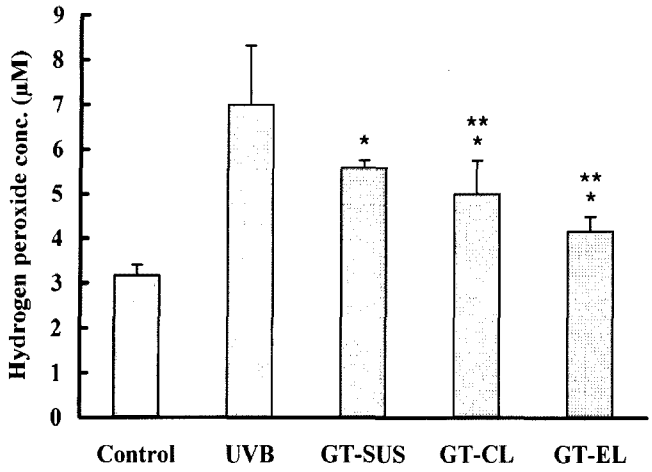

Figure 2–Inhibition effect of GT formulations on UVB-induced hydrogen peroxide production in hairless mouse skin. The bar represents S.D. and statistical analysis was performed using the Student's t-test (*P<0.05 versus UVB; **P<0.05 versus GT-SUS). Control: without any treatment; UVB: UVB treated; GT-SUS: GT-SUS pre-treated; GT-CL: GT-CL pre-treated; GT-EL: GT-EL pre-treated.

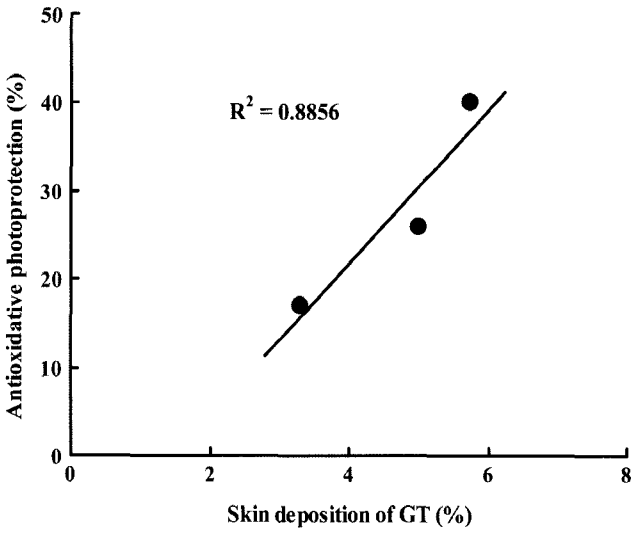

Figure 3–The correlation between skin deposition and antioxidative photoprotection effect for GT-containing gel formulations.

with certain amount of GT prior to UVB exposure could reduce the level of H_2O_2 produced. This effect is counted for the antioxidative activity of GT, in other words, photoprotection effect *in vivo*.

In this experiment, as shown in Figure 2, exposure to UVB for 1 week significantly augmented the production of H_2O_2 in the mouse skin, showing the result of approximately 2.2-fold increase in H_2O_2 level. But topical application of various GT formulations significantly inhibited the production of H_2O_2. Positive control group (pre-treatment of GT-SUS) elicited 17 % inhibition on H_2O_2 production. However, pre-treatment with liposomal formulations of GT-CL and GT-EL further reduced H_2O_2 production for about 26 and 40% inhibition, respectively. As a result, EL revealed the greatest photoprotective effect, alike to the result of skin deposition.

Correlation between skin deposition and photoprotection *in vivo*

The influence of liposomes and suspension on skin deposition and photoprotection was observed as same order of effectiveness. Thus, in order to find a correlation between two observations, the degree of antioxidative photoprotection effect versus the amount of GT deposited in the skin was plotted in Figure 3.

As the increment of GT accumulated in the skin, photoprotection effect was increased. Comparative linearity was found, yielding correlation coefficient (r^2) of 0.8856. This suggests that the amount of soybean isoflavone retained in the skin *in vivo* status is closely correlated with *in vivo* antioxidative activity of GT. Correspondently, it was confirmed that the inhibition activity of GT was dose-dependent in the UVB-induced skin carcinogenesis in animal model.[3)]

Conclusions

GT was successfully incorporated into liposomal nanocarriers and the skin deposition and photoprotection effect were studied *in vivo*. EL composed of soybean phosphatidylcholine and sodium deoxycholate brought the greatest skin deposition of GT by virtue of the properties of both the deformability of the vesicle bilayer and the existence of an osmotic gradient across the skin. Additional *in vivo* study for inhibition effect on UVB-induced H_2O_2 production also revealed the best result with elastic liposomal gel formulation. Linear correlation between skin deposition and photoprotection effect was established. Therefore it might be concluded that elastic liposomes could be a superior nanocarrier system for enhanced skin delivery of GT.

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