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Enhanced transdermal deposition and characterization of quercetin-loaded ethosomes

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Abstract−We sought to evaluate the transdermal permeation potential of quercetin-loaded ethosomes. Quercetin-loaded ethosomes were prepared and characterized with regard to particle size, loading efficiency, stability, and *in vitro* skin permeation. The optimized formulation of ethosomes was confirmed using 2% egg phosphatidylcholine and hydrated 20% ethanol. After quercetin was applied using this formulation, the stability of the ethosomes was determined when loaded with up to 0.04% quercetin. We observed that loading efficiency was improved with increasing concentrations of quercetin. Ethosomes loaded with 0.04% quercetin showed both the greatest loading efficiency (63.9%± 6.0%) and an optimal size range (132±32 nm). Ethosomes loaded with quercetin were superior in skin permeation ability (29.5±7.0 μg/cm^2) compared to either ethanolic solution or liposomes. Therefore, we concluded that quercetin-loaded ethosomes increased the skin delivery of quercetin. Our results suggest that quercetin-loaded ethosomes may enhance the effect of cosmetic materials.

Key words: Ethosomes, Quercetin, Transdermal, Skin Permeation Studies, Cosmetics

INTRODUCTION

A major function of the skin is that of a barrier against the delivery of materials and molecules to the body [1]. This is the main function of the stratum corneum [2,3], which is composed of layers of keratin primarily consisting of corneocytes, and a lipophilic matrix that occupies the empty space between the layers. As the lipids in the stratum corneum consist of a lipid bilayer [1], drug delivery vesicles with lipid bilayer structures that are similar to the biomembrane should be developed to facilitate the delivery of their active components into the skin.

To address this issue, liposomes have been devised as a transdermal drug delivery system. Because liposomes consist of phospholipid biomaterials, they are very bio-friendly and are capable of delivering many active components into the skin [3,4]. Consequently, liposomes have been widely used in the fields of medicine and cosmetics [5,6]. However, traditional liposomes are little or no value as carriers for transdermal drug delivery because they do not deeply penetrate the skin and are accumulated at the stratum corneum [7,8]. Recently, novel vesicular carriers have been developed in an attempt to resolve these problems. Recent approaches in modulating drug delivery through the skin have resulted in the design of two novel vesicular carriers, deformable liposomes and ethosomes [6,9]. Ethosomes are phospholipid vesicles with a high concentration of ethanol. Ethanol is known to be an efficient permeation enhancer. Ethosome vesicles have been reported to have a high attraction to the skin and increased membrane fluidity compared to liposomes. As a result, ethosomes have been shown to be effective in the delivery of active components deep in the skin [8,10-12].

The skin is exposed to attack by various environmental factors such as UV rays [13], pollution, and bacteria. Reactive oxygen species (ROS) are generated by these environmental stressors. These ROS activate proteases to produce oxidative stress in the skin, and thus promote aging induced by damage to collagen, elastin, and DNA. The oxidative stress mediated through these proteases is controlled through an antioxidant barrier system [14-17]. This antioxidant barrier system consists of both enzymatic and nonenzymatic antioxidants for the prevention of aging due to ROS. Of these, there are many natural nonenzymatic antioxidants. In particular, the flavonoids are the most common natural compounds. These flavonoids have multiple physiological properties including anti-inflammatory, antioxidant, and skin whitening [18,19]. Flavonoids have also been shown to have stronger antioxidant activity than either vitamin E or vitamin C [20]. Quercetin is one of the flavonoids involved in the elimination of oxidative radicals. In this role, quercetin has been shown to prevent oxidative damage and cell death, mitigate lipid peroxidation, and chelate transition metal ions in the skin that contribute to aging via continuous oxidative damage [21-25].

Despite these many advantages, flavonoids such as quercetin are known to have water poor solubility. To effectively deliver flavonoids such as quercetin to the skin, a delivery system is necessary. Therefore, the purpose of this study was to determine whether the natural antioxidant quercetin could be efficiently loaded and stably prepared in ethosomes for delivery to the skin. Our results suggest that quercetin-loaded ethosomes can enhance the effects of cosmetic materials.

EXPERIMENTAL

1. Materials

L-α-Phosphatidylcholine from egg yolk (egg PC, ~60%) and quercetin were obtained from Sigma (USA). The solvents used, such as ethanol and chloroform, were of extra pure grade. Preparation of ethosomes required the use of a rotary evaporator (BUCHI, Switzerland) and a probe sonicator (BRANSON, USA). Ethosome diameter was measured using an ELS-Z (Otsuka, Japan). Skin per-

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meation studies used a Franz diffusion cell kit (PermeGear, USA) including a 9 mm Franz diffusion cell (receptor volume 5 mL) and V6A model stirrer. The ethosome loading efficiency and quercetin skin permeation was calculated by using HPLC (Shimadzu, Japan).

2. Preparation of Ethosomes

Ethosomes were prepared according to the thin-film hydration method [10,26-28]. Egg PC (0.5-5%, w/v) and quercetin (0.005-0.05%, w/v) were dissolved in chloroform in a 50 mL round-bottomed flask. The mixture was evaporated in a rotary evaporator and solvent traces were removed. The film was hydrated with ethanolic solution (20-40% ethanol, v/v) at above the lipid transition temperature for 1 h. The vesicle suspension was dispersed using a probe sonicator at 30 min.

3. Measurement of Ethosome Vesicle Size

Vesicle size in ethosome suspensions was measured with an ELS-Z instrument. Particles suspended in liquids are in Brownian motion due to random collisions with solvent molecules. The diffusion coefficient is inversely proportional to the particle size according to the Stokes-Einstein equation. Through dynamic light scattering, the fluctuations in the intensity of scattered light from particles in Brownian motion over can be measured. As particles in Brownian motion move about randomly, the scattered intensity fluctuations are random. The fluctuations of the scattered light were analyzed by using an autocorrelation function. The CONTIN method [29] was used to resolve particle size distributions from measured autocorrelation functions. The CONTIN method takes into account the weighting of the distribution due to the use of discrete data points in the continuous distribution and then calculates different moments of the computed distribution.

4. Measurement of Ethosome Loading Efficiency

Free quercetin was removed from the ethosome suspension with a 1.2 μm filter (Minisart; CA, 26 mm). Ethosome vesicles were disturbed with 15 mL ethanol and then the ethanol was evaporated in a rotary evaporator. The remained quercetin was redissolved in ethanol. The quercetin content was measured by HPLC at 370 nm (the maximum absorption wavelength of quercetin in ethanol solution). To correct the free quercetin content in the filtrate, the same amount of quercetin was dissolved in ethanol and filtered using a 1.2 μm filter. The quercetin content of the filtrate was measured by HPLC at 370 nm. The following Eq. (1) was used to calculate the loading efficiency.

$$\text{Loading efficiency } (\%) = \{(C_P - C_F)/C_0\} \times 100 \quad (1)$$

C_P: content of quercetin passed through the 1.2 μm filter
C_F: content of free quercetin dissolved in the filtrate
C_0: initially added quercetin content

5. *In Vitro* Skin Permeation Studies

Skin permeation studies were used to determine the effect of increasing skin permeation of quercetin in the ethosomal delivery system. Outbred albino ICR mice (8 weeks old, female) were used. Mouse skin, including epidermis and dermis, was taken from the dorsal surface. Experiments were run in Franz diffusion cells with a receptor compartment volume of 5 mL. The receptor compartment contained a receptor phase (HCO-60 : ethanol : phosphate buffered saline (PBS)=2 : 20 : 78 [w/w/w %]) and skin was fixed between the donor and the receptor phase of the stratum corneum side facing upward into the donor compartment. The temperature was maintained at 37 °C [30]. Samples were applied to the skin in the donor compartment. Samples were withdrawn through the sampling port of the diffusion cell at 2, 4, 8, 12, and 24 h. The receptor phase was immediately replenished with an equal volume of fresh receptor phase. The quercetin in withdrawn samples was analyzed by HPLC at 370 nm.

The amount of quercetin retained in skin was determined at the end of the *in vitro* permeation experiment (24 h). The skin was washed three times with PBS. The remaining skin was analyzed for quercetin quantity in the stratum corneum by tape stripping [31]. After tape stripping, the skin was cut into small sections using scissors. The tape and skin were added to 10 mL ethanol. The solution was evaporated in a rotary evaporator using a hydrated receptor phase. The quercetin quantity was then analyzed by HPLC at 370 nm.

6. Statistical Analysis

All reported data are presented as mean±S.E.M. Statistical significance was determined by Student's *t*-test.

RESULTS AND DISCUSSION

1. Vesicle Size of Empty Ethosomes

To determine the concentration of ethanol and egg PC required for optimal stability, ethosomes were prepared using a range of concentrations of ethanol and egg PC (Table 1). When ethosome suspensions were prepared using 20%, 30%, or 40% ethanol, ethosomes were formed at 20% and 30% ethanol. When ethosome suspensions were prepared using 1%, 2%, 3%, or 5% egg PC, ethosomes were formed at 1% and 2% egg PC. When ethosome suspensions were prepared using 3% egg PC, they only formed when using 20% ethanol. But, these results conflicted with previously reported [32,33]. In this study, ethosomes have increased viscosity of formulation with increasing ethanol amount due to the interaction ethanol molecule, phospholipid and water molecules. We supposed that the higher the ethanol amount, the lower the ethosome bilayer stability because of viscosity. The formulation using 2% egg PC and 20% ethanol showed the smallest vesicle size, confirming that this was the optimal formulation. Thus, this formulation was used in subsequent experiments.

2. Quercetin-loaded Ethosomes

2-1. Vesicle Size of Ethosomes

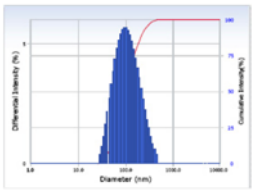
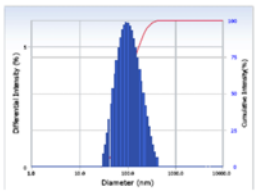
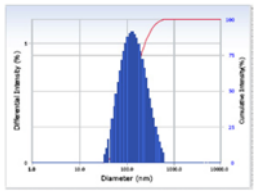
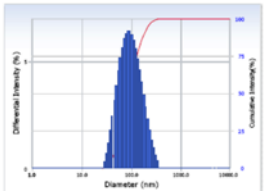
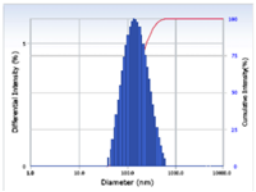
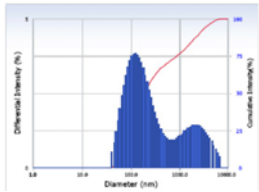
When ethosomes were prepared using 20% ethanol, they were stable until loaded with 0.04% quercetin (Table 2). Ethosomes loaded with 0.05% quercetin appeared not monodisperse and increased the instability of the vesicle bilayer. Therefore, the optimal quercetin concentration for stability of the ethosome bilayer was deter-

Table 1. Mean particle size for different ethanol and egg PC concentrations

	20% Ethanol	30% Ethanol	40% Ethanol
1% Egg PC	160.9±6.2 nm	186.0±11.8 nm	N.S.
2% Egg PC	148.6±6.9 nm	197.0±7.0 nm	N.S.
3% Egg PC	303.6±15.0 nm	N.S.	N.S.
5% Egg PC	N.S.	N.S.	N.S.

Each value is presented as mean±S.D
N.S.: not stable

Table 2. Size of ethosomal vesicles with different concentration of quercetin

Concentration of quercetin (%)	0.005	0.01	0.02
Size of ethosome (nm)			
	121.0±15.8	126.5±2.0	164.8±0.8
Concentration of quercetin (%)	0.03	0.04	0.05
Size of ethosome (nm)			
	126.8±12.8	132.2±32.1	198.5±26.5 2735.2±638.0

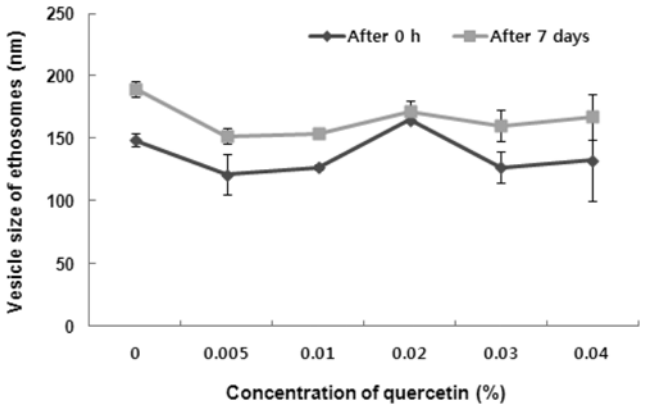


Fig. 1. Vesicle sizes and stability of ethosomes over seven days with different quercetin concentrations in a system composed of 2% lecithin and 20% ethanol.

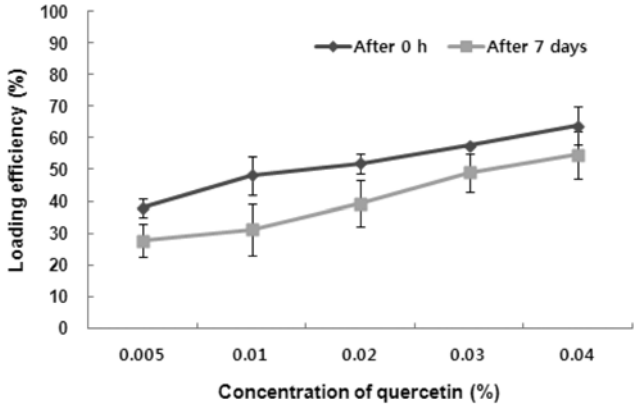


Fig. 2. Loading efficiency of ethosomes according to concentration of quercetin.

mined to be below 0.04%. Immediately after preparation, vesicle sizes were determined to be 120-160 nm (Fig. 1). The vesicle size of quercetin-loaded ethosomes was measured, and the results showed that with increasing amounts of quercetin, there was a concomitant increase in vesicle size. These results also indicated more quercetin in the ethosome. According to initial findings, vesicle size was predicted to be smallest for unloaded ethosomes. However, in contrast to our prediction, vesicle size was smaller in quercetin-loaded ethosomes than in unloaded ethosomes. To determine the stability of the loaded ethosomes, we measured the vesicle size 1 week after preparation. After this time period, the vesicle size had increased 4.1-26.5% (Fig. 1). Despite this increase, the ethosomes were deemed to be stable because the vesicles remained intact and the ratio of the vesicle size increase was small.

2-2. Loading Efficiency

Fig. 2 indicates the loading efficiency of different quercetin-loaded ethosomal formulations. Loading efficiency was determined to be 37.07±3.94%, 48.22±6.03%, 51.91±3.01%, 57.62±0.01% and 63.90±6.00% for 0.005% to 0.04% quercetin-loaded ethosomes. These results indicated that the greater the concentration of loaded quercetin, the greater the loading efficiency. Ethosome loading efficiency was also measured one week after preparation. These results indicated that the stability of the loading efficiency remained above 70%. The loading efficiency of the 0.04% quercetin-loaded ethosomal formulation was optimal. Therefore, this loading concentration was used in subsequent experiments.

2-3. *In Vitro* Skin Permeation Studies

The ethosomal system used for the skin permeation studies consisted of hydrated 20% ethanol and quercetin loaded at 0.04%. The control groups used liposomes loaded with 0.04% quercetin, 0.04% quercetin in hydrated ethanolic solution, and 0.04% quercetin in hydrated distilled water. The skin permeation of quercetin during 24 h after applied systems and solutions was shown to be the highest for the ethosomal system (3.6±0.3 μg/cm^2), followed by the liposomal system (2.3±0.4 μg/cm^2), the ethanolic solution (1.4±0.4 μg/cm^2), and finally, distilled water (0.6±0.2 μg/cm^2) (Fig. 3). Thus, the transdermal skin permeation studies indicated that the ethosome and liposome systems using phospholipid were superior for the trans-

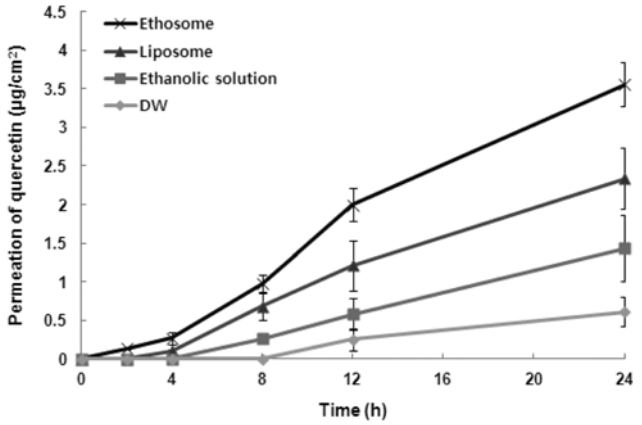


Fig. 3. Comparative cumulative amount of transdermal quercetin permeation from ethosomes, liposomes, ethanolic solution, and distilled water (DW) solution over 24 h into dorsal mouse skin.

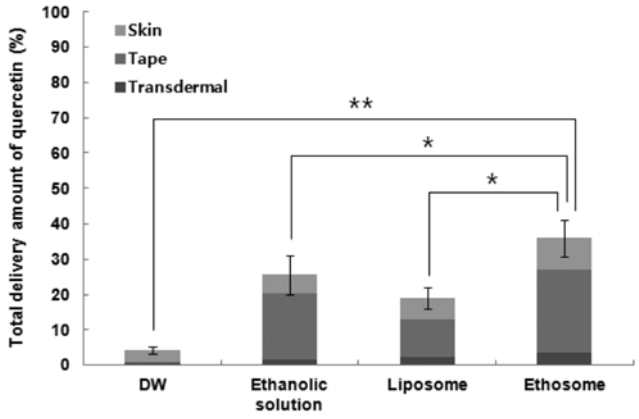


Fig. 5. Total delivery amount of quercetin from ethosomes, liposomes, ethanolic solution, and distilled water (DW) solution over 24 h into dorsal mouse skin (Tape : stratum corneum, skin : dermis and epidermis without stratum corneum, transdermal: permeated through skin). *p<0.05, ** p<0.01.

dermal delivery of quercetin, and that of the two systems, the effects were superior in the ethosome system. The amount of quercetin deposited in the stratum corneum was determined to be as follows: ethosome (18.8±0.6 μg/cm^2)>ethanolic solution (15.1±1.7 μg/cm^2)>liposome (8.6±1.9 μg/cm^2)>distilled water (0.1±0.02 μg/cm^2) (Fig. 4). The quercetin deposited in the stratum corneum confirmed the effect of ethanol on the enhancement of skin delivery. In the skin layers outside the stratum corneum, the amount of quercetin deposited was found to be: ethosome (7.1±2.9 μg/cm^2)>liposome (4.8±1.1 μg/cm^2)>ethanolic solution (4.3±0.4 μg/cm^2)>distilled water (2.8±0.3 μg/cm^2) (Fig. 4). Our results also showed that the total penetration of quercetin followed a similar pattern as the stratum corneum: ethosome (29.5±7.0 μg/cm^2)>ethanolic solution (20.8±4.5 μg/cm^2)>liposome (15.7±2.0 μg/cm^2)>distilled water (3.6±0.8 μg/cm^2). The amount of quercetin used for the skin permeation studies was 80 μg/cm^2 in every system. The penetration ratios for the ethosomes, ethanolic solution, liposomes, and distilled water were 36.9%, 26.0%, 19.6%, and 4.5%, respectively (Fig. 5). Our *in vitro* skin permeation studies confirmed the effect of phospholipids and ethanol in ethosomes. The amount of transdermal quercetin permeation was superior in ethosomes and liposomes; therefore, we suspect that the phospholipid effects were responsible for the enhanced penetration of quercetin deep in the skin. As the deposition of quercetin in the skin was superior by ethosomes and ethanolic solution, we propose that ethanol disrupted the barrier effect at the stratum corneum. Collectively, our data suggest that ethosomes act through a combination of effects via both phospholipids and ethanol. Ethosomes were confirmed to be the best system for the delivery of quercetin to the skin by virtue of this complementary function.

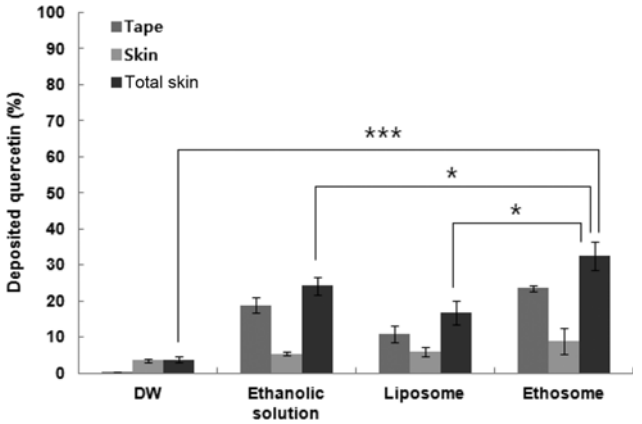


Fig. 4. Deposition of quercetin measured from ethosomes, liposomes, ethanolic solution, and distilled water (DW) solution over 24 h into dorsal mouse skin. *p<0.05, ***p<0.005.

CONCLUSIONS

Quercetin, a naturally occurring compound, has high antioxidant activity but poor water solubility. To address this problem, ethosomes were used to deliver quercetin to the skin. The optimized formulation for ethosomes was determined to be 2% egg PC and hydrated 20% ethanol. When quercetin was loaded into ethosomes using this formulation, they were found to be stable up to a concentration of 0.04%. For an estimation of skin permeation for the optimized formulation of ethosomes, we performed *in vitro* skin permeation studies. The results of these studies showed that ethosomes are superior in the delivery of quercetin to the skin compared to liposomes, ethanolic solution, and distilled water. Therefore, quercetin-loaded ethosomes present a solution to the problems associated with quercetin delivery to the skin. After considering all of these results, we suggest that quercetin-loaded ethosomes can enhance the effect of cosmetic materials.

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