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Enhancing Skin Antioxidant Delivery and Efficacy: Evaluating Liposomal Formulations of Resveratrol, Coenzyme Q10, and Vitamin E

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Abstract: ***This study aims to evaluate the influence of liposomes on the effective delivery of antioxidants into the skin. A liposomal cream(RSV-CoQ10-VitE-Lipo)and a non-liposomal cream (RSV-CoQ10-VitE-Cream)containing resveratrol, coenzyme Q10, vitamin E were prepared. The particle size (PS), zeta potential (ZP), polydispersity index (PDI) and encapsulation efficacy (EE) of liposomes were determined. In vitro permeation studies of the formulations were performed with porcine skin in Franz cells over 48 hours. Antioxidant activities of samples were measured with DPPH assay. RSV-CoQ10-VitE-Lipo had mean size 96.4 nm, PDI 0.292, ZP -49.7 mV, 95.375% RSV, 95.885% CoQ10, 95.683% VitE. DPPH assay results showed that RSV-CoQ10-VitE-Lipo had the highest antioxidant activity (90.36% ± 1.1).RSV-CoQ10-VitE-Lipo is an effective topical formulation for protecting skin against free radicals.***

Keywords: Delivery, liposome, resveratrol, coenzyme Q10, topical

1. Introduction

Reactive oxygen species (ROS) are free radicals that are produced specifically through cell oxidative metabolism in the course of adenosine triphosphate (ATP) generation from glucose. Ultraviolet (UV) radiation causes high generation of ROS. ROS accumulation in cells cause oxidative damage and mitochondrial dysfunction which lead to chronological aging. [1, 2, 3, 4, 5, 6]. Antioxidants are active substances that can prevent the occurrence and reduce the severity of UV-induced skin damage and skin aging [7, 8]. Resveratrol (RSV) is a potent inhibitor of UV-induced lipid peroxidation [9]. It is a poorly water-soluble drug (<0.001 mol/l), poorly absorbed after oral administration, and it converts to the cis-form (a less active form), especially when exposed to UV light [10, 11, 12]. Coenzyme Q10 (CoQ10), an endogenous antioxidant, stimulates cell growth and scavenges free radicals [13]. Vitamin E (VitE) is an antioxidant that inhibits lipid peroxidation [14]. It plays an important role in the formation of the lipid structure of stratum corneum and in the protection of proteins from oxidation [15].

Liposomes are vesicular carrier systems that can encapsulate both hydrophilic and lipophilic substances [16, 17, 18, 19]. They can penetrate the skin easily due to their membranes being very similar to the lipids of the stratum corneum [20]. By the encapsulation of unstable active substances into liposomes, their shelf-life and bioavailability can be improved and they can be transported into deeper layers of skin [21, 22, 23]. Arora et al. developed an anti-aging resveratrol-loaded vesicular gel and demonstrated that encapsulating resveratrol in vesicular gel resulted in higher antioxidant activity than plain gel of resveratrol [24]. Lee et al. indicated that encapsulation of CoQ10 into liposomes enhanced its permeation through rat skin [25]. Previous studies demonstrate that antioxidant liposomes exert much better protection against tissue damage due to oxidative stress compared to free, non-encapsulated antioxidants. Although there are examples of liposomal antioxidant formulations on the market, a topical liposomal formulation containing resveratrol, coenzyme Q10 and vitamin E in combination has not been developed so far [26, 27, 28, 29]. Antioxidants when used together in the same formulation enhance antioxidant activity of each other [30, 31, 32]. It has been reported that resveratrol and antioxidant vitamins work better together than any of them on their own for protection against free radicals [31]. In addition, CoQ10 and VitE enhance the antioxidant activity of each other when they are combined together. CoQ10 and VitE work together to counteract prooxidant effects of ROS. In the membrane lipid bilayer, CoQ10 can recycle α-tocopherol (VitE) from the tocopheroxyl radical, prevent VitE loss and lipid peroxidation [32]. Therefore, encapsulation of RSV, CoQ10 and VitE together into a liposomal formulation could be effective to obtain high antioxidant protection against ROS and photoaging.

The aim of the present study was to develop a liposomal formulation that contains the combination of antioxidants resveratrol, coenzyme Q10 and vitamin E, to characterize the liposomal formulation in terms of structure, particle size, zeta potential, and polydispersity index and to evaluate its skin delivery and antioxidant activity compared to a non-liposomal cream formulation.

2. Materials & Methods

2.1 Materials

Transresveratrol, coenzyme Q10, alfa-tocopherol, cholesterol (Ch), soy phosphatidylcholine (SPC), HCO-60, dipalmitoylphosphatidylcholine(DPPC), DPPH• radical, all high-performance liquid chromatography (HPLC) reagents and chloroformwere purchased from Sigma, USA. The

other chemicals were obtained from Merck KGaA (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.2 Assay

The quantitative determination of RSV was carried out by high performance liquid chromatography (HPLC) method. The HPLC equipment used is consisted of a Shimadzu SIL-20AC Ht HPLC autosampler and pump. Inertsil ODS-3V 250 x 4.60 mm 3 µm column was used for the analysis. Methanol: water (51:49, v/v) was used as the mobile phase. The flow rate of mobile phase was adjusted to 0.9 ml/min. The detection wavelength was set to 306 nm.

For the quantitative determination of CoQ10 and VitE with HPLC, Hypersil GOLD 250 x 4.60 mm 5 µm column was used. Methanol:n-hexane (80:20, v/v) was used as mobile phase in isocratic mode. The flow rate of mobile phase was adjusted to 1 ml/min. The detection wavelength was set to 275 nm.

2.3 Preparation of the liposomes

Soy lecithin (SPC), cholesterol (Ch) (1:1, w/w) and the active ingredients (1%, w/v) were dissolved in a mixture of chloroform/methanol (2:1 v/v). The solution was transferred into a round-bottom flask. The organic solvents were removed by a rotary evaporator (Rotavapor-R, W. Büchi, Flawil Schweiz). The lipid film was hydrated with distilled water. The suspension was agitated by vortex for 5 minutes and left in an ultrasonic bath for 15 minutes. Then, the liposome was sonicated with a probe sonicator for 10 minutes to reduce the vesicle size.

2.4 Preparation of the cream formulations

Beeswax, paraffin and spermaceti were melted in a water bath. Almond oil was added and the mixture was heated to 70 ºC. Borax and methyl paraben were dissolved in water and heated to 70 ºC. After both phases had reached 70 ºC, the aqueous phase was slowly added to the melted oil phase with stirring. The cream was allowed to cool slowly with constant stirring. The liposomal suspension was incorporated into the cream in such a way that the final formulation (RSV-CoQ10-VitE-Lipo) contained 1% w/w RSV, 1% w/w CoQ10 and 1% w/w VitE. Similarly, a non-liposomal formulation (RSV-CoQ10-VitE-Cream) (containing 1% w/w RSV, 1% w/w CoQ10 and 1% w/w VitE) using the same base was prepared.

2.5 Determination of Formulation Characteristics

Particle size (PS), zeta potential (ZP) and polydispersity index (PDI) of formulations were determined usinga Zetasizer Nano ZS-Malvern.

2.6 Drug Entrapment Efficacy

The loaded liposomes were centrifuged and the encapsulation efficiency (EE) was calculated from the supernatant by a validated HPLC method using the equation below:

$$EE\% = \frac{Total\ amount\ of\ drug\text{-}amount\ of\ unloaded\ drug}{Total\ amount\ of\ drug} \times 100 \quad (1)$$

2.7 *In vitro* Skin Permeation Study

To determine the skin permeation, Franz diffusion cells were used. Porcine skin obtained from a local butcher was used in the study. The temperature of the cells was kept constant at 37°C. HCO-60: ethanol: PBS (2:20:78, v/v/v) solution was used as the receptor solution. The skin was first washed with water, then the hairs and fat layers were removed gently. Afterwards, the skin was cut into smooth pieces of approximately 800 µm thickness and stored at -70°C. Before use, it was taken from the freezer and kept at room temperature for a while, then kept in the receptor solution for 30 minutes, and was placed in Franz diffusion cells. The skin was placed between the two halves of the cell, with stratum corneum facing the donor compartment. There was no air space between them. The remaining parts were cut. The receptor compartment had a volume of 11 mL. 2 ml of each formulation was placed to the donor compartment. After 1, 2, 4, 8, 12, 24, 48 hours, 1 ml of the receptor solution was taken and the active substance content was determined by the adopted HPLC method. The cumulative amount of drug permeated was plotted as a function of time.

2.8 *In vitro* Evaluation of Antioxidant Activity

For antioxidant activity, DPPH● radical scavenging activity was measured spectrophotometrically (Cary 60 UV-Vis spectrophotometer, Agilent, USA). The DPPH radical scavenging activity of RSV-CoQ10-VitE-Lipo and RSV-CoQ10-VitE-Cream were determined. This comparison was performed to examine the effect ofliposomes on the bioavailability of the antioxidants.

The DPPH scavenging effects of natural or synthetic compounds are determined by removing the purple color of the 2,2-diphenyl-1-picrylhydrazil stable radical. In this method, the color of the compounds with the DPPH radical is measured at 517 nm. It is then compared with the standard substance. When a substance that can give off a hydrogen atom and a solution of DPPH are mixed, the purple color disappears and turns into the reduced form. DPPH-H stands for reduced form and R● stands for free radical formed in the first step. This radical then reacts as many times as the number of DPPH molecules that are reduced (decolorized) by a reducing molecule [51]. For antioxidant activity studies, 500 µL sample was prepared in 1.5 mL of 0.1 mM methanol. It was then mixed with the DPPH● solution. It was incubated for 30 minutes at room temperature and in the dark. The absorbances were read at 517 nm. The percent inhibition was calculated by the equation below. Analyzes were performed in three parallels and mean values were used.

$$Inhibition\ \% = \frac{Absorbance\ of\ control\text{-}Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100 \quad (2)$$

2.9 Statistical Analysis

All data in this study were considered as means ± SD and one-way ANOVA was used for statistical analysis. GraphPadInStatver.2(CA,USA) was used.

3. Results & Discussion

Resveratrol, coenzyme Q10 and vitamin E are antioxidant compounds that can be used for anti-aging effects by neutralizing the free radicals that cause photoaging of skin. However, their low solubility and low oral bioavailability limit their use [10-14]. Liposomes can encapsulate the quickly degradable compounds like RSV, CoQ10 and VitE. Previous studies have shown that encapsulation of the antioxidants with liposomes can improve bioavailability [33, 34, 35]. Therefore, a liposomal formulation of RSV, CoQ10 and VitE was developed, its topical delivery and antioxidant activity was evaluated and compared with a non-liposomal cream containing the combination of the same antioxidants.
In this study, RSV-CoQ10-VitE-Lipo was successfully prepared by thin film hydration method. The quantitative determination of RSV, CoQ10 and VitE in the formulations was carried out by a validated HPLC method.

Characterization of the liposomes plays an important role for understanding the quality of the liposomes. Particle size and polydispersity index are important parameters for the characterization of vesicles due to their effect on efficacy, stability and safety. It has been reported that large particles have difficulty reaching the desired location within the target tissue since they are not able to diffuse through the deeper skin layers [36]. The liposomes were found to have particle size 96.4 ± 0.02 nm, zeta potential -49.7 ± 0.2 mV and polydispersity index 0.292 ± 0.01. It has been established in previous studies that for delivery into deeper layers of skin, the particle size of liposomes must be smaller than 300 nm since liposomal formulations with larger particle size stay on the stratum corneum and they tend to dry forming a lipid layer on the skin. Vesicles smaller than 300 nm can deliver the actives into deeper layers of skin and are advantageous for dermal drug delivery [37, 38]. Nava et al. and Verma et al. indicated that vesicular carrier systems with smaller particle size penetrate through the skin more efficiently than the vesicles with larger particle size [37, 39]. RSV-CoQ10-VitE-Lipohad a particle size smaller than 300 nm confirming its potential for effective delivery through the skin. Chen et al. developed CoQ10 loaded liposomes with mean diameter 166 nm [40]. Layas et al. reported that resveratrol-loaded liposomes revealed a size range between 197.4 ± 102.4 and 457.9 ± 56.1 nm [41]. Vijayakumar et al. developed liposomes containing resveratrol and vitamin E with particle size range from 125.5 ± 9.25 nm to 134.2 ± 9.13 and demonstrated that increasing the sonication time led to changes in the particle size during the preparation of liposomes containing trans-resveratrol and D-α-tocopherol [42]. The particle size of liposomes in the present study were below 100 nm which may be attributed to the higher length of the sonication time.

Zeta potential is a parameter for the evaluation of the stability of liposomes. Vanaja et al. reported that colloidal systems with a zeta potential value greater than +30 mV and less than -30 mV are stable [43]. In the present study, the liposomal formulation had a negative surface charge which indicates that the liposomal dispersion has a homogeneous distribution and it is stable. The negativity of zeta potential may be attributed to the presence of phospholipids since the functional groups of phospholipids are negatively charged. The liposomal dispersions with more than 30mV zeta potential are considered as stable, and liposomes with less than 30 mV tend to agglomerate because of low electrostatic repulsion and the overcoming van der Walls interactions. If the zeta potential is below 30 mV, the attractive forces overcome the repulsive electrostatic forces and cause agglomeration [43]. Vijayakumar et al. developed liposomes containing resveratrol and vitamin E with zeta potentials between -37.26 ± 1.16 mV and -64.35 ± 0.16 mV, and poly dispersity index between 0.182 ± 0.03 and 0.265 ± 0.01 [42]. Gokce et al. indicated that CoQ10 loaded liposomes had zeta potential −36.6 ± 0.9 mV and PDI 0.458 ± 0.03 [44]. Regarding the zeta potential measurements of the present study, the liposomal formulation has a zeta potential within the acceptable range which indicates that the characterization results of the investigated formulation were in accordance with the previously published data. The PDI of the newly developed liposomal formulation was below 0.3, which indicates the homogeneity of the liposomes.

The drug entrapment efficacy of RSV was 95.375 % ± 0.01, of CoQ10 was 95.885 % ± 0.02 and of VitE was 95.683 % ± 0.05 in the liposomes. Vanaja et al. have developed RSV and VitE liposomes with encapsulation efficacies >90% and >79, respectively [43]. In the present study, the drug entrapment efficacy values are higher than 90 which proves that the antioxidants are successfully encapsulated into liposomes. *In vitro* diffusion test of RSV, CoQ10, and VitE in the formulations was performed using Franz diffusion cell. RSV-CoQ10-VitE-Lipo and RSV-CoQ10-VitE-Cream were used as the donor phases. After 1, 2, 4, 8, 12, 24, 48 hours, a sample from the receptor solution was taken and the active substance content was determined by HPLC. The cumulative penetrated amounts of RSV, CoQ10 and VitE from the formulations through the membrane were plotted as a function of time (Fig.1, Fig.2, Fig.3).

The skin acts as a barrier to the penetration of active substances [45]. For topical permeation studies, several animal models are in use as natural membranes in order to predict the skin penetration of actives. Animal models that have direct comparability with human skin or similarity to topical absorption in humans are chosen to evaluate the penetration of active compounds. Porcine skin is widely preferred because it has many similarities to human skin (fat deposits, follicles, density) and is easily accessible [46, 47, 48]. In the present study, for the determination of the *in vitro* skin permeation, porcine skin was placed between the donor and receptor compartments of Franz diffusion cells. HCO-60: ethanol: PBS (2:20:78, v/v/v) solution was used as the receptor solution. Since RSV, CoQ10 and VitE have low water solubility, HCO-60 and ethanol were used to enhance the penetration of active compounds. 2 ml of the formulation was placed to the donor compartment. After 1, 2, 4, 8, 12, 24, 48 hours, a 1 ml sample from the receptor solution was taken and the active substance content was determined by a validated HPLC method. The cumulative penetrated amounts of RSV, CoQ10 and VitE from the formulations through porcine skin were plotted as a function of time (Fig. 2). The permeation of the RSV, CoQ10 and VitE from RSV-CoQ10-VitE-Cream was lower than the liposomal cream. This can be attributed to the enhanced

skin-penetrating ability of liposomes. Due to their similarities in lipid composition and structure with biological membranes, liposomes enhance the penetration of the entrapped actives [16, 18]. After 48 hours, there was a significant drop in the permeated amount of active ingredients from RSV-CoQ10-VitE-Cream. On the contrary, after 48 hours, the permeated amounts of RSV, CoQ10 and VitE were higher than 500 mcg/cm^2 showing that the antioxidants encapsulated in liposomes can pass through the skin successfully. This infers that liposomes improve the release of active substances by providing a deposit of them in the skin. Liposomes act as a reservoir for the continuous delivery of actives and provide local deposition of active compounds on the action site [16, 18].

For antioxidant activity, DPPH● radical scavenging activity was measured spectrophotometrically and the antioxidant effect of each formulation was determined (Table 1). Nashine et al. and Trotta et al. reported that resveratrol formulations showed scavenging activities of more than 50% of DPPH free radicals [49, 50]. Cervellati et al. reported that the antioxidant activity of CoQ10 was higher than 50% [51]. In the present study, both liposomal and non-liposomal cream formulations were able to scavenge more than 50% of the DPPH● radical after 24 hours. The results were in accordance with the previously published data. The radical scavenging activity of RSV-CoQ10-VitE-Lipo was significantly higher than the non-liposomal cream which can be attributed to the enhanced antioxidant activity achieved by encapsulating the antioxidants.

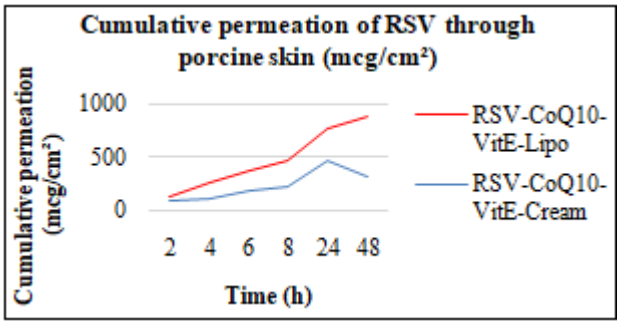

Figure 1: Cumulative permeation of RSV through the porcine skin (mcg/cm²)

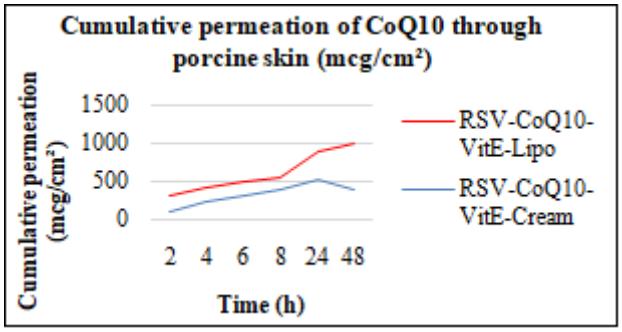

Figure 2: Cumulative permeation of CoQ10 through the porcine skin (mcg/cm²)

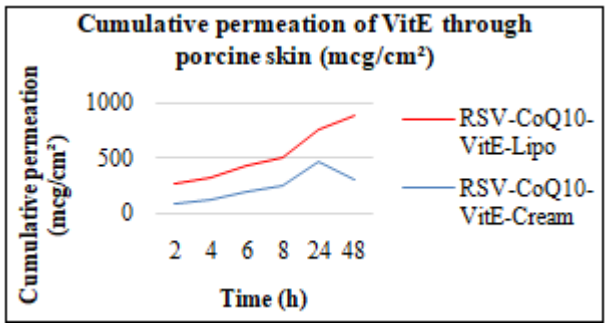

Figure 3: Cumulative permeation of VitE through the porcine skin (mcg/cm²)

Table 1: Antioxidant activities of formulations (n=3)

Formulations	DPPH (Inhibition %) ±SD
RSV-CoQ10-VitE-Lipo	90.36% ± 1.1
RSV-CoQ10-VitE-Cream	55.48% ± 1.7

*Data are expressed using mean value ±standard deviation, *n*=3

4. Conclusion

A liposomal formulation that contains RSV, CoQ10 and VitE was successfully formulated and characterized. DLS measurements clearly indicated that RSV-CoQ10-VitE liposomes were uniform. It has been indicated in the previously published studies that vesicles with particle size smaller than 300 nm can deliver the actives into deeper layers of skin [37, 38]. In the present study, the particle size of all the liposomal formulations were smaller than 300 nm confirming that the liposomes can penetrate into deeper layers of skin efficiently. It has been previously reported that increasing the sonication time resulted in smaller vesicle size [42]. In our study, the vesicle size was three folds smaller than the published data since the sonication time is higher. The encapsulation efficacies of the liposomes were higher than the previously published data. The liposomal formulation can encapsulate RSV, CoQ10 and VitE successfully. The particle size, zeta potential and PDI of liposomes showed that the liposomes were stable. The permeated amounts of RSV, CoQ10 and VitE were highest from the RSV-CoQ10-VitE-Lipo than RSV-CoQ10-VitE-Cream. Liposomes increased the skin penetration of active compounds significantly. The newly developed RSV-CoQ10-VitE-Lipo formulation have been found to bemuch more efficient than RSV-CoQ10-VitE-Cream forskin delivery. In several studies, it has been reported that RSV formulations and CoQ10 showed scavenging activities of more than 50% of DPPH free radicals [49, 50, 51]. As expected, in the present study, improved antioxidant activity was observed by encapsulating the antioxidants RSV, CoQ10 and VitE together in the liposomes. The liposomal cream formulation that contains RSV, CoQ10 and VitE is effective for protecting skin against free radicals and slowing down skin aging by topicalapplication.

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