Microemulsion Using Polyoxyethylene Sorbitan Trioleate and Its Usage for Skin Delivery of Resveratrol to Protect Skin against UV-Induced Damage

Reiko Yutani,* Reiko Teraoka, and Shuji Kitagawa

Kobe Pharmaceutical University; 4–19–1 Motoyamakita-machi, Higashinada-ku, Kobe 658–8558, Japan.

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We examined the phase behavior of various polyoxyethylene sorbitan fatty acid ester (polysorbates)/ethanol/isopropyl myristate (IPM)/150 mM NaCl solution (NaClaq) systems in order to prepare a microemulsion containing a low ratio of ethanol, which is more suitable for in vivo application. Using polyoxyethylene sorbitan trioleate (Twee 85), which has a large lipophilic moiety, as a surfactant component, single-phase domain of the phase diagram was the largest of all the polysorbates examined, and in particular a large oil-rich single-phase domain was obtained. When the ratio of Tween 85 to ethanol was changed from 1:1 to 3:1, the oil-rich single-phase domain further expanded, which led to a reduced ethanol concentration in the preparation. Thus, we determined the composition of the microemulsion to be Tween 85:ethanol:IPM:NaClaq=30:10:53:7, and used it for skin delivery of resveratrol. Microemulsion gel was also prepared by adding 6.5% Aerosil® 200 into the microemulsion for ease of topical application. When applied with each vehicle, delivery of resveratrol into guinea pig skin in vitro was significantly enhanced compared with that by IPM, and resveratrol incorporated into the skin by microemulsion gel decreased lipid peroxidation to 29.5% compared with that of the control. Pretreatment of guinea pig dorsal skin with the microemulsion gel containing resveratrol almost completely prevented UV-B-induced erythema formation in vivo. These findings demonstrate that the microemulsion using Tween 85 containing a minimal concentration of ethanol enhanced the skin delivery of resveratrol and the incorporated resveratrol exhibited a protective effect against UV-induced oxidative damage.

Key words resveratrol; microemulsion; polyoxyethylene sorbitan trioleate; skin delivery; photoprotection; phase diagram

Resveratrol (trans-3,5,4′-trihydroxystilbene, Fig. 1) is a naturally occurring polyphenolic phytoalexin synthesized by a wide variety of plant species, including grapes, berries and peanuts, in response to environmental stress and microbial infection.1,2 It has been suggested in several studies that resveratrol is a potent antioxidant with anti-inflammatory and antiproliferative properties1–5; thus, the application of resveratrol for topical purposes has been examined. However, since the penetration of resveratrol into skin is limited,6 the use of an enhancement system is an effective way of facilitating the penetration and the exertion of its physiological activities in skin.

To improve the skin delivery of resveratrol, we have used microemulsion as an enhancement system.6) Microemulsions consist of an aqueous phase, an oil phase, a surfactant and a co-surfactant component, which are thermodynamically stable and have been shown to have high solubilization capacity and to facilitate the skin incorporation of both hydrophilic and lipophilic drugs.7,8) In previous studies, we revealed that a microemulsion consisting of polyoxyethylene sorbitan monooleate (Twee 80), ethanol, isopropyl myristate (IPM) and 150 mm NaCl solution (NaClaq) at a weight ratio of 30:30:33:7 improved the intradermal delivery of polyphenols, such as quercetin,9 genistein10 and chlorogenic acid,11) and the polyphenols incorporated into skin prevented lipid peroxidation8,10) and UV-induced erythema formation.10,11) This microemulsion also increased the incorporation of resveratrol into skin, compared with that by IPM.6)

In this study, we first attempted to prepare a microemulsion containing a lower ratio of ethanol, which is more suitable for in vivo application to avoid skin irritancy. Pseudo-ternary phase diagrams using various polyoxyethylene sorbitan fatty acid esters (polysorbates) were constructed and the composition of the microemulsion was determined. Microemulsion gel was also prepared using Aerosil® 200 as a gelling agent, to retain the preparation at the site of application for practical use. Next, intradermal and transdermal delivery of resveratrol was examined in vitro using guinea pig skin, and the anti-lipoperoxidative activity of resveratrol incorporated by microemulsion gel was assessed by the thiobarbituric acid (TBA) test. Furthermore, the effect of resveratrol incorporated by microemulsion gel on UV-B-induced erythema formation was examined in vivo using guinea pigs.

Results and Discussion

We first examined the phase behavior of various polysorbate/ethanol/IPM/NaClaq systems. Pseudo-ternary phase dia-
grams using different polysorbates as a surfactant component are presented in Fig. 2. The upper part of the plots shown in gray on the diagrams represents the single-phase domain. As shown in Figs. 2(a)–(d), the single-phase domain slightly expanded with increasing hydrophobicity of the fatty acid chain (polyoxyethylene (20) sorbitan monolaurate (Tween 20)<poly-
The composition of the microemulsion using Tween 80 (Tween 80 microemulsion, depicted with a star in Fig. 2(i)), by modifying receptor compartment of IPM.

Thus determined the composition of the microemulsion to be 30 : 30 : 33 : 7 (Tween 85 : ethanol : IPM : NaCl). For decreasing the ratio of ethanol in a preparation, we examined the oil-rich single-phase domain of all the polysorbates, which are assumed to form large interfacial surfaces in the microemulsion, enabling the incorporation of greater amounts of drug molecules. Since the increased solubility in the vehicle does not necessarily lead to increased skin delivery of the drug, penetration enhancers seem to be involved in the mechanism of action of topical application of resveratrol with the microemulsion gel on UV-induced damage, we examined the antiliperoxidative activity of resveratrol incorporated into skin by microemulsion gel. In terms of the results, the incorporated resveratrol significantly inhibited lipid peroxidation: the production of TBA-reactive substances decreased to 29.5% compared with that for the control gel without resveratrol.

To clarify the effects of topical application of resveratrol with the microemulsion gel on UV-induced damage, we examined the antiliperoxidative activity of resveratrol incorporated into skin by microemulsion gel. In terms of the results, the incorporated resveratrol significantly inhibited lipid peroxidation: the production of TBA-reactive substances decreased to 29.5% compared with that for the control gel without resveratrol.

To confirm the antioxidative effect of resveratrol in skin, we then examined the antiliperoxidative activity of resveratrol incorporated into skin by microemulsion gel. In terms of the results, the incorporated resveratrol significantly inhibited lipid peroxidation: the production of TBA-reactive substances decreased to 29.5% compared with that for the control gel without resveratrol.

The open bars represent the amount of resveratrol incorporated into the skin and the shaded bars represent the amount of resveratrol that permeated into the receptor compartment. Data are mean ± S.D. of at least 3 values. ***p < 0.001, compared with the data in the skin of IPM; *p < 0.001, **p < 0.01, compared with the data in the receptor compartment of IPM.

We next examined the intradermal and transdermal delivery of resveratrol to assess the drug delivery potential of Tween 85 microemulsion. Microemulsion gel was also prepared by adding 6.5% Aerosil® 200 into Tween 85 microemulsion for the ease of topical application. As shown in Fig. 3, both Tween 85 microemulsion and microemulsion gel significantly improved the skin delivery of resveratrol at both time points compared with that by IPM. The amounts of incorporation in skin by the microemulsion and that by microemulsion gel after 20 h of application were about 8-fold and 7-fold higher than that by IPM, respectively. Permeation of resveratrol into the receptor compartment was found for all vehicles, but the amount of permeation was smaller than the amount of incorporation in skin.

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enhancement by microemulsions.\textsuperscript{7,8)}

UV-B, which is an essential component of sunlight, can cross the whole epidermis layer and penetrate the upper dermis, resulting in erythema, edema, sunburn cells, hyperplasia, immunosuppression, photoaging and photocarcinogenesis, which are directly or indirectly related to the excessive production of reactive oxygen species.\textsuperscript{1,3,12)} It has been demonstrated that photo-damage can be reduced when the skin is supplemented with antioxidants.\textsuperscript{3,12)} In this study, resveratrol incorporated into skin by microemulsion gel containing a low ratio of ethanol effectively prevented against UV-B-induced erythema formation, as revealed for genistein and chlorogenic acid in previous studies.\textsuperscript{10,11)} This effect of resveratrol is attributable to its strong photoprotective properties, for example, the reduction of reactive oxygen species, the inhibition of cyclooxygenase and the decreased infiltration of leukocytes.\textsuperscript{1,3,4,12,13)}

**Conclusion**

The results obtained from the phase diagrams demonstrated that Tween 85, which has a large lipophilic moiety, exhibited a large oil-rich single-phase domain. We thus chose Tween 85 as a surfactant to prepare a microemulsion containing a low ratio of ethanol, and confirmed the photoprotective effect of resveratrol incorporated into skin by the microemulsion gel. The findings obtained in this study demonstrated that Tween 85 microemulsion would be useful for enhancement of the skin delivery of resveratrol to protect the skin against UV-induced oxidative damage.

**Experimental**

**Materials**

Resveratrol and trans-ferulic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Tween 20, Tween 40, Tween 80, Tween 60, Tween 85, IPM, ethanol and 2-thiobarbituric acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Tween 21, Tween 61 and Tween 65 were kindly provided by Croda Japan KK (Tokyo, Japan). Aerosil® 200 was purchased by Nippon Aerosil Co., Ltd. (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dorsal skin excised from Hartley male guinea pigs, whose fur on the back had been cut using hair clippers beforehand, was purchased from Japan SLC, Inc. (Hamamatsu, Japan), and Hartley male guinea pigs were also obtained.

**Pseudo-ternary Phase Diagram Construction**

Pseudo-ternary phase diagrams were constructed at room temperature. NaClaq was used as an aqueous phase, IPM as an oil phase, one of the polysorbates (Tween 20, Tween 40, Tween 80, Tween 60, Tween 85, Tween 21, Tween 61, Tween 65 and Tween 85) as a surfactant component and ethanol as a co-surfactant component. The surfactant and co-surfactant were mixed at a weight ratio of 1 : 1 for all polysorbates and 3 : 1 for Tween 85. At each ratio, 1 g of surfactant/co-surfactant/oil phase mixture was prepared at weight ratios from 1 : 9 to 9 : 1 ((surfactant/co-surfactant) : oil phase), and then titrated with 10 µL of aqueous solution and mixed using a vortex mixer. This procedure was repeated until the transparent dispersion became translucent or turbid and the weight ratio at this time was plotted on the phase diagrams. To estimate the whole single-phase domain, surfactant/co-surfactant/aqueous phase mixtures were also prepared, and the boundary of the single-phase domain was determined.

**Preparation of Microemulsion and Microemulsion Gel**

Tween 85 microemulsion was obtained by vortex mixing and resveratrol was added to it at a concentration of 200 µmol resveratrol applied to the left side (control site, which is shown in the lower part of each picture) of the guinea pig dorsal skin and microemulsion gel with 200 µmol resveratrol was applied to the right side (resveratrol-preloaded site). (c) represents the difference of a* values between before and after irradiation (Δa*): Res (−), control site; Res (+), resveratrol-preloaded site. Data are mean ± S.D. of 3 values. Measurements were performed at 3 points at each site.

**In Vitro Study on Skin Delivery of Resveratrol**

In vitro studies on skin delivery of resveratrol were performed to determine the efficacy of the microemulsion gel in protecting the skin against UV-induced oxidative damage. The microemulsion gel was applied to the left side (control site) and the right side (resveratrol-preloaded site) of the dorsal skin of guinea pigs. The Δa* values were measured at 3 points at each site, and the results were compared to determine the efficacy of the microemulsion gel in protecting the skin against UV-induced oxidative damage.
Lipid Peroxidation Assay  The anti-lipoperoxidative activity of resveratrol incorporated into skin by microemulsion gel was evaluated in vitro by the formation of malondialdehyde (MDA), as described previously. After 20h of treatment, microemulsion gel containing 200 µmol resveratrol with skin as described above, the skin was homogenized in 1.15% KCl solution. Ammonium iron(II) sulfate and sodium citrate were added to 1.0 mL of sample solution containing about 0.2 mg of protein to final concentrations of 50 µM and 2 mM, respectively, and the solution was kept for 30 min at 37°C. Then, 0.20 mL of 8.1% sodium dodecyl sulfate solution, 1.5 mL of acetate buffer adjusted to pH 3.5, 50 µL of 0.8% butylhydroxytoluene in glacial acetic acid, 1.5 mL of 0.8% TBA solution, and 0.7 mL of 5 mM iron(III) chloride solution were added in this order, followed by 60 min of incubation at 60°C. After cooling, MDA-TBA complex was extracted with 5 mL of butanol and pyridine mixed solution, centrifuged at 1670 g for 10 min. The samples were then centrifuged and the supernatants were used for determination by HPLC. The receptor solution was also collected after treatment to determine the amount of resveratrol that had permeated through the skin by HPLC.

Observation of Erythema Formation  Erythema formation induced by UV irradiation was observed as described previously, following the protocol approved by the Animal Experimentation Committee of Kobe Pharmaceutical University. Fur on the dorsal skin of guinea pigs was cut using hair clippers and shavers and dirt on the skin was washed off. Approximately 24h later, microemulsion gel containing 200 µmol resveratrol was applied to the right side of the dorsal skin, while microemulsion gel without resveratrol was applied to the left side. After approximately 24h, microemulsion gel remaining on the skin surface was wiped off with wet gauze repeatedly. After drying the skin surface for about 5h, both sides of the skin were irradiated for 24 min using UV light at an intensity of 138 µW/cm² measured with a UV-Meter 340 (Ando Keiki Co., Ltd., Tokyo, Japan). UV-B lamps, GL20SE (Sankyo Denki Co., Ltd., Kanagawa, Japan), emitting a continuous light spectrum between 280 and 380 nm with peak emission at 306 nm, were used. Erythema formation was evaluated from photographs of the back of the guinea pigs as well as measurement of the skin redness (a*) with a color reader, CR-13 (Konica Minolta, Inc., Tokyo, Japan).

Statistical Analysis  The results are expressed as mean±standard deviation (S.D.) of at least 3 values. Data were analyzed by one-way ANOVA followed by Dunnett’s test. Differences were regarded as significant at p<0.05.

Conflict of Interest  The authors declare no conflict of interest.

References