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The effect of oil components on the physicochemical properties and drug delivery of emulsions: Tocol emulsion versus lipid emulsion

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Abstract

An emulsion system composed of vitamin E, coconut oil, soybean phosphatidylcholine, non-ionic surfactants, and polyethylene glycol (PEG) derivatives (referred to as the tocol emulsion) was characterized in terms of its physicochemical properties, drug release, in vivo efficacy, toxicity, and stability. Systems without vitamin E (referred to as the lipid emulsion) and without any oils (referred to as the aqueous micelle system) were prepared for comparison. A lipophilic antioxidant, resveratrol, was used as the model drug for emulsion loading. The incorporation of Brij 35 and PEG derivatives reduced the vesicle diameter to <100 nm. The inclusion of resveratrol into the emulsions and aqueous micelles retarded the drug release. The in vitro release rate showed a decrease in the order of aqueous micelle system > tocol emulsion > lipid emulsion. Treatment of resveratrol dramatically reduced the intimal hyperplasia of the injured vascular wall in rats. There was no significant difference in this reduction when resveratrol was delivered by either emulsion or the aqueous micelle system. The percentages of erythrocyte hemolysis by the emulsions and aqueous micelle system were ∼0 and ∼10%, respectively. Vitamin E prevented the aggregation of emulsion vesicles. The mean vesicle size of the tocol emulsion remained unchanged during 30 days at 37 °C. The lipid emulsion and aqueous micelle system, respectively, showed 11- and 16-fold increases in vesicle size after 30 days of storage.

Keywords: Tocol emulsion; Lipid emulsion; Aqueous micelle; Resveratrol; Drug delivery

1. Introduction

The parenteral administration of lipophilic drugs is a major problem in pharmaceutical formulation design. Lipid emulsions are attractive candidates for improving drug solubility. They are well accepted for their ability to incorporate lipophilic drugs, to reduce side effects of various potent drugs, to increase the bioavailibility of drugs, and to prolong the pharmacological effects in comparison to conventional formulations (Youenang Piemi et al., 1999). The oils typically used for pharmaceutical emulsions consist of digestible oils from natural sources. There is a need for novel, biocompatible formulations which are cost-effective, non-irritating, and capable of being sterilized before application. The preferred formulation should also have an acceptable shelf-life, and accommodate a wide variety of water-insoluble and poorly soluble drugs. Alternative biocompatible oils such as vitamin E (α-tocopherol) and/or other tocols have recently been investigated for drug delivery (Constandinides et al., 2004). A tocol emulsion was used to provide a safe, lipophilic component to solubilize a highly water-insoluble molecule, paclitaxel, without the use of toxic organic solvents (Constandinides et al., 2000). Vitamin E may also enhance the anticancer action of doxorubicin (Ripoll et al., 1986). Vitamin E has been shown to prevent amiodarone-induced injury of human endothelial cells because of its antioxidant effect (Kachel et al., 1990), which makes a tocol-based emulsion of amiodarone very advantageous. Vitamin E may also allow for higher drug doses without increasing toxicity (Weijl et al., 1997).

Although there are many advantages of using vitamin E as an alternative oil in emulsion systems, the benefits of frequent vitamin E uptake are controversial (Patterson et al., 1999). Some sizeable randomized trials of antioxidant vitamins have shown that vitamin E cannot reduce mortality due to cardiovascular death or cerebrovascular accidents (Vivekananthan et al., 2003).

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The aim of this study was to explore whether or not the incorporation of vitamin E in emulsions has some advantages. A lipid emulsion without vitamin E was used for comparison. Resveratrol was used as the model drug delivered by the emulsions. Resveratrol, a natural product from red wine, can play an important role in the prevention of and therapy for cardiovascular diseases and cancers (Frémont, 2000). The oral bioavailibility of resveratrol is poor, leading to an irrelevant in vivo effect by oral administration as compared to its powerful in vitro efficacy (Goldberg et al., 2003). Hence, other routes such as a parenteral injection should be considered in order to obtain better therapeutic benefits.

Resveratrol shows a similar solubility profile to that of paclitaxel (Kan et al., 1999; Hung et al., 2006). The solubility of resveratrol in pH 7.4 buffer is only 13.6 μg/g. Hence, resveratrol may be suitable to be incorporated into emulsions. A synergistic effect on the inhibition of vascular intimal thickening was examined when treating emulsions with resveratrol and vitamin E. The physicochemical properties, in vitro drug release, toxicity, and storage stability of the developed emulsions were evaluated. The formulation without oil composition (the aqueous micelle system) was also used in these evaluations.

2. Materials and methods

2.1. Materials

Vitamin E, coconut oil, glycerol formal, Span 80, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical (St. Louis, MO, USA). Hydrogenated soybean phosphatidylcholine (Phospholipon® 80H) was obtained from American Lecithin Company (Oxford, CT, USA). Brij 30 and Brij 35 were supplied by Acros Organics (Geel, Belgium). Tween 80 was from Showa Chemicals (Tokyo, Japan). The polyethylene glycol (PEG) derivative of distearoylphosphatidylethanolamine (PEP; with a mean molecular weight of PEG: 2000) and cholesterol (CP; with a mean molecular weight of PEG: 2000) were purchased from Nippon Oil (Tokyo, Japan). Cellulose membranes (Cellu-Sep® T2, with a molecular weight cutoff of 6000–8000) were supplied by Membrane Filtration Products (Seguin, TX, USA).

2.2. Preparation of emulsions

Coconut oil (5%, w/v), vitamin E (5%), soybean phosphatidylcholine (5%), and/or non-ionic surfactants (3%) were dissolved in an appropriate volume of chloroform:methanol (2:1). In the formulation without vitamin E (the lipid emulsion), the percentage of coconut oil was 10%. The organic solvent was evaporated in a rotary evaporator at 50 °C until a thin film was formed on the surface of a flask, and solvent traces were removed by maintaining the lipid film under a vacuum for 6 h. The films were hydrated with a glycerol formal:water solution (1:9) containing resveratrol (0.2% of the resulting product) by a high-shear homogenization (homogenizer: Pro Scientific Pro 250, Monroe, CT, USA) for 10 min at 60 °C. Then the resulting solution was sonicated using a probe-type sonicator (Sonics and Materials VCX 600, CT, USA) for 30 min at 60 °C. The intensity of the ultrasound was set at 35 W. The percentage of typical composition in formulations is shown in Table 1.

Table 1
The percentage (%) of composition of tocol emulsion, lipid emulsion, and aqueous micelles

Composition[a]	Tocol emulsion	Lipid emulsion	Aqueous micelles
Coconut oil	5	10	0
Vitamin E	5	0	0
Glycerol formal	10	10	10
Phospholipon® 80H	5	5	5
Water	74.8	74.8	84.8
Resveratrol	0.2	0.2	0.2

[a] The co-emulsifiers or PEG derivatives with a percentage of 3 and 1% instead of water, respectively, were added if necessary.

2.3. Vesicle size and zeta potential

The mean particle size (*z*-average) and zeta potential of the emulsions were measured by a laser scattering method (Malvern Nano ZS® 90, Worcestershire, UK). The formulations were diluted 100-fold with double-distilled water before the measurement. The determination was repeated three times/sample for three samples. The stability was determined by monitoring the size of a 1-ml formulation in the presence of 2 ml of normal saline. The long-term stability was determined at 37 °C as a function of time for 30 days (in the absence of normal saline).

2.4. In vitro drug release

Resveratrol release from the emulsions was measured using a Franz diffusion cell. The cellulose membrane was mounted between the donor and receptor compartments. The donor medium consisted of 1 ml of vehicle containing resveratrol. The receptor medium consisted of 10 ml of 30% ethanol in pH 7.4 buffer to maintain the sink condition during the experiments. The available diffusion area between cells was 1.767 cm^2. The stirring rate and temperature were kept at 600 rpm and 37 °C, respectively. At appropriate intervals, 300-μl aliquots of the receptor medium were withdrawn and immediately replaced with an equal volume of fresh buffer. The released amount of drug was determined by HPLC.

The HPLC system (Hitachi, Tokyo, Japan) consists of a 25-cm-long, 4-mm inner diameter stainless C18 column (Merck, Darmstadt, Germany) and a mobile phase of methanol and water at pH 2.7 adjusted by acetic acid (45:55). The flow rate and UV wavelength were set at 1 ml/min and 310 nm, respectively.

2.5. Rat balloon injury study

Adult male Sprague–Dawley rats weighing 400–500 g were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Each animal was anesthetized by an intraperitoneal injection of chloral hydrate (40 mg/kg), followed by a longitudinal midline cervical incision that permitted exposure of the left

common, external, and internal carotid arteries. A 2F catheter was introduced through the external carotid artery into the common carotid artery. Then the balloon was inflated, and moved in and out three times without rotation along the common carotid artery. After deflating the balloon, the catheter was withdrawn, the external carotid artery was ligated, the neck incision was closed, and the rats were returned to their cages.

The control group of rats was treated daily for 7 days prior to and 14 days following the balloon injury by intraperitoneal injections of saline. The formulations with resveratrol (1 mg/kg) were also administered via intraperitoneal injections. Fourteen days after the balloon injury, animals were anesthetized and sacrificed with an intraperitoneal injection of chloral hydrate. The left carotid arteries were removed, fixed in 4% formaldehyde, and stained with hematoxylin and eosin (H&E) for light microscopy in a standard manner. The neointimal area/medial area ratio was quantitatively measured using image microscopy (Olympus BX51) and image measurement software (SPOT Application). Each experiment was performed triplicate.

2.6. Radical scavenging activity

DPPH, a stable free radical, was used to determine the radical scavenging activity of resveratrol, vitamin E, and the emulsions. A 15-μl formulation was added to 1.5 ml ethanol with 100 μM DPPH at 25 °C. The mixture was stirred with a stirring bar and maintained for 15 min in the dark; the absorbency was then measured at 517 nm. The results are expressed as a percentage of DPPH reduced by various formulations.

2.7. Erythrocyte hemolysis

Blood samples were obtained from a healthy donor by venipuncture and collected into test tubes containing 124 mM sodium citrate (1 volume of sodium citrate solution + 9 volumes of blood). The erythrocytes were immediately separated by centrifugation at 2000 × *g* for 5 min and washed three times with 4 volumes of a normal saline solution. Erythrocytes collected from 1 ml of blood were resuspended in 10 ml of normal saline. Immediately thereafter, 2.5 ml of 2% (w/v) dispersions of the formulations and mixtures thereof in saline were incubated with 0.1 ml of the erythrocyte suspension. Incubations were carried out at 37 °C with gentle tumbling of the test tubes. After 1 h of incubation, the samples were centrifuged for 5 min at 2000 × *g*. The absorbance of the supernatant was measured at 415 nm to determine the percentage of cells undergoing hemolysis. Hemolysis induced with double-distilled water was taken as 100%.

2.8. Statistical analysis

The statistical analysis of differences among the various treatments was performed using unpaired Student's *t*-test. A 0.05 level of probability was taken as the level of significance. An ANOVA test was also used if necessary.

3. Results and discussion

3.1. Physicochemical properties

Vegetable oils are generally well accepted for parenteral administration. Coconut oil (5%, w/v) and vitamin E (5%, w/v) were used as the oil phases of the tocol emulsion for resveratrol. Coconut oil was selected because it had the highest solubility for resveratrol (179.8 μg/g) among the vegetable oils examined (Hung et al., 2006). The reason for incorporating glycerol formal in the emulsions was that glycerol formal produced great solubility of resveratrol (Sale et al., 2004). We had examined resveratrol solubility in glycerol formal and found a value of >5000 μg/g (Hung et al., 2006).

Soybean phosphatidylcholine cannot be used as an emulsifier alone because it does not produce emulsions over a wide range of oil and water compositions (Brime et al., 2002). It was thus necessary to incorporate co-emulsifiers into the emulsion systems. As shown in Table 2, the tocol emulsion without co-emulsifiers (plain emulsion) revealed a droplet size of ∼180 nm. The addition of co-emulsifiers either increased or decreased the droplet size. Span 80 and Brij 30 significantly increased ($p < 0.05$) the droplet size to ∼210 and ∼300 nm, respectively. On the other hand, the incorporation of Tween 80 and Brij 35 to the tocol emulsion led to an initial decrease in the particle size. The polydispersity index could be well controlled to a range of 0.20–0.35

Table 2
The composition and characterization of tocol emulsions by vesicle size and zeta potential

Additives of emulsion[a]	Size (nm)	Zeta potential (mV)	Polydispersity index
Plain emulsion	183.4 ± 3.8	−67.6 ± 0.3	0.23
+Span 80 (3%)	213.3 ± 7.3	−75.8 ± 3.6	0.21
+Tween 80 (3%)	153.4 ± 3.3	−52.4 ± 0.6	0.20
+Brij 30 (3%)	299.2 ± 1.8	−79.7 ± 0.8	0.35
+Brij 35 (3%)	97.3 ± 2.3	−56.6 ± 3.9	0.27
+Brij 35 (3%) and PEP (1%)[b]	82.9 ± 0.7	−45.1 ± 0.4	0.28
+Brij 35 (3%) and CP (1%)[c]	94.9 ± 1.2	−42.9 ± 0.6	0.30
+Brij 35 (3%), PEP (1%) and CP (1%)	101.3 ± 0.9	−37.5 ± 1.9	0.31

Each value represents the mean ± S.D. ($n = 3$).
[a] The ratio of the additives is weight ratio (%).
[b] PEP, distearoyl phosphatidylethanolamine-PEG 2000.
[c] CP, cholesterol-PEG 2000.

(Table 2). The incorporation of Span 80 and Tween 80 did not largely affect the polydispersity. On the other hand, Brij surfactants could increase this value, especially for Brij 30. PEG derivatives had a trend to enhance the size distribution, presumably due to the crumpled shell morphology and non-spherical shapes of the PEG-containing particles (Borden et al., 2004).

The hydrophile–lipophile balances (HLBs) of Span 80, Brij 30, Tween 80, and Brij 35 were 8.0, 9.7, 15.0, and 16.9, respectively. This suggests that the more-hydrophilic co-emulsifiers (Tween 80 and Brij 35) contributed to a smaller-sized droplet. One parameter for the surfactant film separating the water and oil domains is the spontaneous mean curvature, H_0. H_0 expresses the natural tendency of the monolayer to bend away from a flat geometry (von Corswant et al., 1998). H_0 is positive for co-emulsifiers with a large polar head group and a small nonpolar group and decreases with the number and size of the alkyl chains of the nonpolar group. Span 80 and Brij 30 may be too highly lipophilic to form stable emulsions. This may partly result in the high polydispersity of the tocol emulsion with Brij 30, although this phenomenon was not observed for Span 80. The addition of a hydrophilic co-emulsifier was required to increase the hydrophilicity of phosphatidylcholine in the films, favoring interfacial film curvature.

Brij 35 reduced the particle size to <100 nm. This is advantageous for tocol emulsions since the emulsions can be sterilized simply by passing them through a sterile syringe-driven filter with no need for thermal treatment (Wang et al., 2002). Another benefit is that the large emulsions rapidly disappeared from the blood. On the other hand, small emulsions (~100 nm) show reduced hepatic uptake and prolonged blood circulation times (Kawakami et al., 2000). A major breakthrough in prolonging the residence time in the body was the coating of vesicles with PEG. The bulky PEG headgroup serves as a barrier preventing interactions with plasma opsonins as a result of the concentration of highly hydrated groups that sterically inhibit hydrophobic and electrostatic interactions of a variety of blood components at the droplet surface, thereby prolonging circulation time (Kawakami et al., 2000; Gabizon et al., 2003). PEP and CP were incorporated into the tocol emulsion for this reason. The addition of PEG did not greatly alter the particle size (Table 2). Although the hydrophilic PEG may reside on the vesicles to produce the larger size, the increase in hydrophilicity of the phosphatidylcholine films may favor interfacial film curvature based on the increase in H_0, thus producing an offsetting effect.

The absolute zeta potential of these tocol emulsions were −40 to −80 mV as shown in Table 2. The anionic fractions such as phosphatidylserine, phosphatidic acid, phosphatidylgycerol, and phosphatidylinositol in soybean lecithin with 80% phosphatidylcholine (Phospholipon® 80H) were responsible for the negative surface charges. Phosphatidylcholine exhibits no net charge at physiological pH levels (Chansiri et al., 1999). The addition of the hydrophilic non-ionic surfactants, Tween 80 and Brij 35, to the film led to initial decreases in the zeta potential. Tween 80 and Brij 35 may reside on the oil/water interface near the aqueous phase because of their hydrophilicity. This may result in a shielding of the negative surface charge provided by the phospholipids. This phenomenon was not observed with lipophilic non-ionic surfactants. Another observation was that the larger-sized tocol emulsion possessed a higher surface potential (Pearson's correlation coefficient = 0.91, $p < 0.001$). The larger surface area in the larger oil droplets may be an explanation for this phenomenon.

In order to explore the role of vitamin E in the emulsions, a formulation without vitamin E (lipid emulsion) was prepared. Besides the formation of oil droplets in the emulsions, it is common in emulsion systems using phosphatidylcholine and non-ionic co-emulsifiers for some micelles and/or liposomes to coexist with the majority of emulsion vesicles (Liu and Liu, 1995; Pongcharoenkiat et al., 2002). Hence the influence of these vesicles on the behaviors of the emulsions cannot be ignored. When 10% glycerol formal was added to the water, no particle size or zeta potential was detected by the laser scanning method. As phospholipids were further added to this system during the manufacturing process, a mean particle size and zeta potential of 96.2 nm and −77.0 mV were determined, indicating the formation of micelles or vesicles. Hence there are four different environments in an oil-in-water emulsion: the oil phase; the oil/water interface; the aqueous micelles and the water (Pongcharoenkiat et al., 2002). The formulation without coconut oil or vitamin E (aqueous micelles) was thus prepared for comparison. As shown in Fig. 1, the particle size decreased in the order of tocol emulsion > lipid emulsion > aqueous micelles in the formulations with or without Brij 35, PEP, or CP. The droplet sizes of emulsions without vitamin E are smaller. The viscosity of vitamin E is higher than that of coconut oil. The additional increase in viscosity leads to an increase in emulsion size (Jumaa and Müller, 1998). In order to elucidate this hypothesis, Brij 35-containing emulsions with vitamin E:coconut oil ratios of 8:2 and 2:8 were also prepared for size determination. The formulations with vitamin E percentages of 0% (lipid emulsion), 2 and 5% (tocol emulsion), and 8% showed mean sizes of 62.2, 62.7,

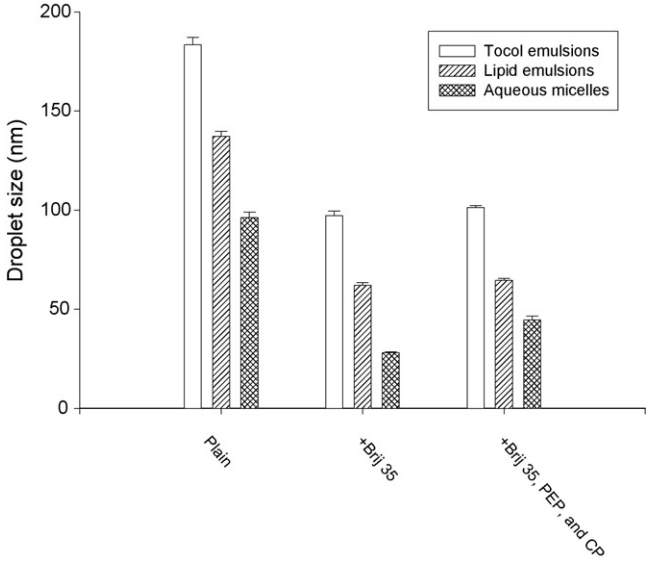

Fig. 1. Droplet sizes of the tocol emulsion, lipid emulsion, and aqueous micelle system with or without the incorporation of Brij 35, PEP, and CP. Each value represents the mean and S.D. ($n = 3$).

97.3, and 233.5 nm, respectively. This indicates the ability of vitamin E to cause size enlargement.

The aqueous micelle system exhibited smaller sizes compared to the emulsions. This suggests that the micelles and liposomal vesicles were smaller than the oil droplets formed by oil and phosphatidylcholine. There were no significant size changes ($p > 0.05$) for the tocol and lipid emulsions after the incorporation of the PEG derivatives. However, the micelle size significantly increased ($p < 0.05$) by intercalating PEG derivatives in the phospholipid bilayers. This is because the H_0 theory only fits the oil/water interface. The size reduction effect by hydrophilic PEG due to interfacial film curvature was absent from the micelle or liposome system. The particle size and zeta potential of the formulations with PEG derivatives (PEP and CP) were also examined in the presence of resveratrol. As shown in Fig. 2, the droplet size is slightly but significantly increased ($p < 0.05$) after encapsulation of resveratrol. This may indicate the inclusion of drug molecules into the oil phase. This increase was especially significant for lipid emulsion. The resveratrol inclusion did not alter the zeta potential of tocol emulsion ($p > 0.05$, Fig. 2). However, this inclusion significantly reduced the surface charge of lipid emulsion and aqueous micelles. This may infer that resveratrol could reside in the oil/water interface or lipid bilayers, shielding the negative charge on the particle surface. Further study is needed

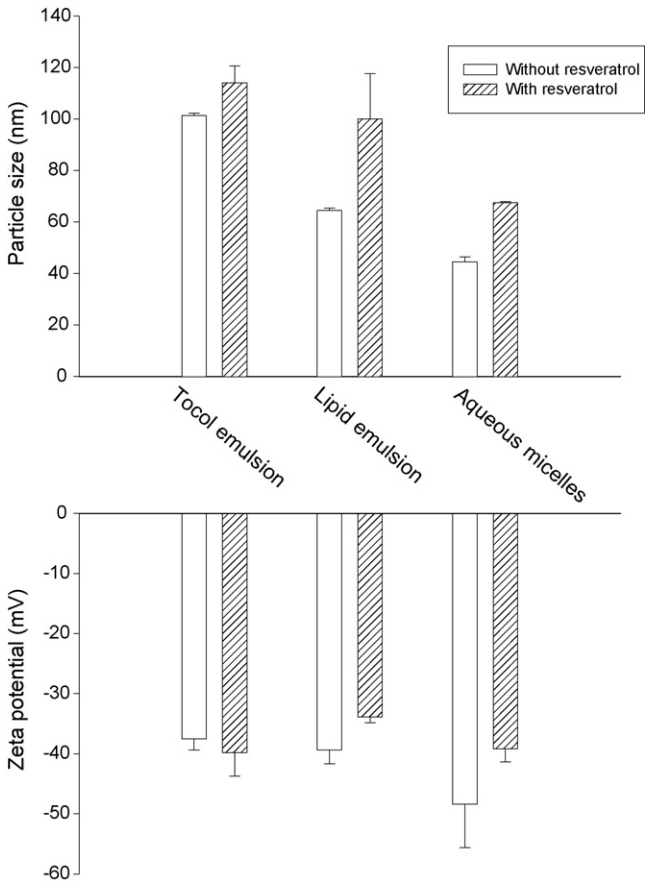

Fig. 2. Droplet size and zeta potential of the tocol emulsion, lipid emulsion, and aqueous micelle system with PEP and CP in the absence and presence of a 0.2% resveratrol. Each value represents the mean and S.D. ($n = 3$).

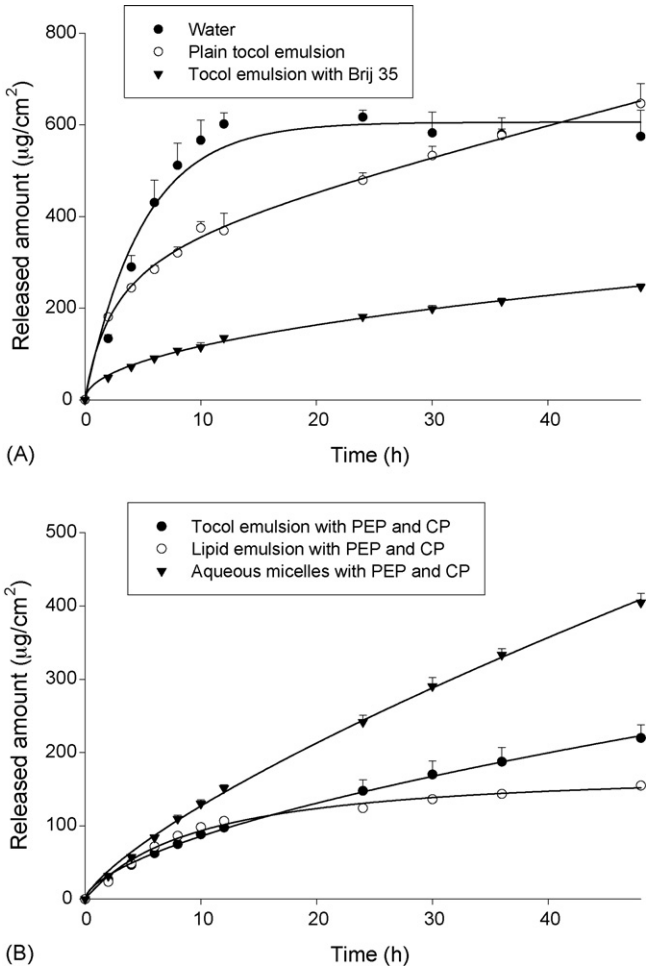

Fig. 3. In vitro release of resveratrol across a cellulose membrane from an aqueous solution (control) and plain or Brij 35-containing tocol emulsion (A), and tocol emulsion, lipid emulsion, and aqueous micelle system with PEP and CP (B). Each value represents the mean and S.D. ($n = 4$).

to explore the mechanisms involving the size and potential charges.

3.2. In vitro drug release

The effect of emulsions and aqueous micelles on resveratrol release was investigated by determining the drug release across a cellulose membrane. Release of resveratrol from water was also studied as a control group. As shown in Fig. 3A, the release of resveratrol from the aqueous solution showed an initial burst, then leveled off after 10 h of administration. There was a ~55% drug dose released from aqueous solution to the receptor phase at steady state. The limited release amount may be due to the use of in vitro Franz cell. Since drugs may release to a definitive space of receptor (10 ml in this study), the drug loading in the receptor is limited. Nevertheless, this method was still useful to differentiate the release capability of various formulations. The plain tocol emulsion retarded drug release and attained the same release amount as the aqueous solution after 30 h ($p > 0.05$). The tocol emulsion incorporating Brij 35 further slowed down the drug release. Resveratrol was gradually released from the Brij

35-containing tocol emulsion for 48 h, indicating that this emulsion could be a carrier for controlled resveratrol delivery. The drug is stably retained in the vesicles for a determined duration, followed by a slow release into the external phase. The incorporation of Brij 35 may further strengthen the oil/water interface, thus reducing the release of resveratrol from oil droplets to the external aqueous phase.

As shown in Fig. 3B, the incorporation of PEP and CP did not significantly change the release profiles of resveratrol from the tocol emulsion. This indicates that the PEG derivatives did not alter the permeability of vesicles in the tocol emulsion. The in vitro release of resveratrol from the PEG-containing formulations showed a decrease in the order of liposome system > tocol emulsion > lipid emulsion. This possibly indicates the higher permeability of the phospholipid bilayers of micelles or liposomes compared to the oil/water interfacial layers. The drug residing in the oil phase may slow down its release. Another possible reason is that the rate of release generally increases from smaller particles since a small-particle system has a large total surface area where drug diffusion can occur (Chung et al., 2001). The particle numbers in the systems (i.e., the count rate; particles counted per second) were also monitored by a light-scattering method. The count rates of the tocol emulsion, lipid emulsion, and liposome system after a 100-fold dilution were 1.19×10^6, 1.33×10^6, and 1.96×10^6 particles, respectively. This value was inversely correlated with the mean vesicle size. Hence, aqueous micelles with a smaller size exhibited a larger particle population, contributing to the larger surface area.

The release profile of the lipid emulsion approximated that of the tocol emulsion during the first 12 h. The resveratrol release from the lipid emulsion was gradually reduced after 12 h compared to that from the tocol emulsion. Coconut oil showed a high solubility for resveratrol. This may have decreased the partitioning of the drug from the oil to the aqueous phase as the release procedure occurred. The release profiles suggested that by altering the composition, the resveratrol release can be well controlled. This is important for the development of a system for use as a drug carrier for parenteral use.

3.3. In vivo efficacy of resveratrol

Resveratrol inhibits the oxidation of low-density lipoprotein (LDL) and the initial stage of the pathogenesis of atherosclerosis, and suppresses the proliferation of smooth muscle cells (Burns et al., 2002). Restenosis is characterized by hyperplasia of the intima, primarily as a result of proliferation of smooth muscle cells embedded in the extracellular matrix of the intima (Liu et al., 1989). In order to examine the therapeutic efficacy of resveratrol in emulsions, an in vivo model to induce restenosis in the rat carotid artery was used. There was a significant increase in the intimal area and a decrease in the cross-sectional area of the lumen in the injured artery, which received the balloon injury and was treated with normal saline only (Fig. 4A and B). The intimal thickening that formed after the endothelial injury contained smooth muscle cells embedded in a matrix. The intimal hyperplasia was inhibited to a substantial degree in the resveratrol-treated groups as shown in Fig. 4C–E. Resveratrol acts as an antioxidant to antagonize the production of free radicals and in turn favorably influences the course of coronary heart disease. Resveratrol significantly inhibits proliferation of smooth muscle cells stimulated by endothelin, angitension II, and serum mitogens (Zou et al., 2000). A complementary action of resveratrol is to suppress platelet adhesion and aggregation (Frémont, 2000).

The neointimal/media ratio is calculated to quantify the inhibitory effect of resveratrol on hyperplasia as shown in Fig. 5. Resveratrol reduced the vascular intimal thickening to a significant level ($p < 0.05$) as compared to the control in the tocol emulsion, lipid emulsion, and aqueous micelles. Differences among the neointimal/media ratio of these formulations were not significant ($p > 0.05$) when the data were subjected to statistical analysis. Although the release profiles of resveratrol from these formulations differed, the final efficacies of the emulsions and micelles were approximately similar. All three formulations examined showed a particle size of <100 nm. Particles at the nanolevel (<100 nm) can show reduced hepatic uptake and a prolonged blood circulation time (Kawakami et al., 2000). The in vivo pharmacodynamic activity of resveratrol in emulsions and aqueous micelles may be predominantly controlled by the size but not the drug release rate.

Another observation is that the incorporation of vitamin E did not synergize the inhibitory effect of resveratrol against hyperplasia. This may have been due to the antioxidants like vitamin E being more efficient in inhibiting the early stages of atherosclerosis (Steinberg and Witztum, 2002). Vitamin E may be inefficient with a severe injury of the capillary wall such as balloon injury. A part of the answer to this dilemma may be provided by the discovery of the so-called "tocopherol-mediated peroxidation" which exemplifies the paradoxical role of vitamin E in the autoxidation of LDL (Schneider, 2005). The results of this present study favor the theory of limited benefits of vitamin E on the already formed neointima, although the advantages of vitamin E are still controversial these days (Vivekananthan et al., 2003).

3.4. Radical scavenging activity

In order to further explore the antioxidant activities of resveratrol and vitamin E, the in vitro scavenging capacity toward DPPH was examined. Resveratrol and vitamin E at the same dose (0.2 and 5.0%) in the tocol emulsion were first dissolved in DMSO to examine their scavenging activities. As shown in Fig. 6, resveratrol exhibited a DPPH reduction ratio of 45%. Vitamin E was very efficient at scavenging DPPH (89%) when present at a concentration of 5.0%. When formulating the tocol emulsion with vitamin E and either with or without resveratrol, the scavenging activity was not altered ($p > 0.05$). However, the scavenging activity of resveratrol was significantly reduced ($p < 0.05$) when formulating the lipid emulsion and aqueous micelle system. This indicates that the inclusion of resveratrol into oil droplets and/or liposomal vesicles may shield the antioxidant activity of the drug. The lipid emulsion and aqueous micelle system without resveratrol showed negligible DPPH reduction.

Vitamin E is the most powerful lipid-soluble antioxidant known, and only recently developed novel synthetic antioxidants

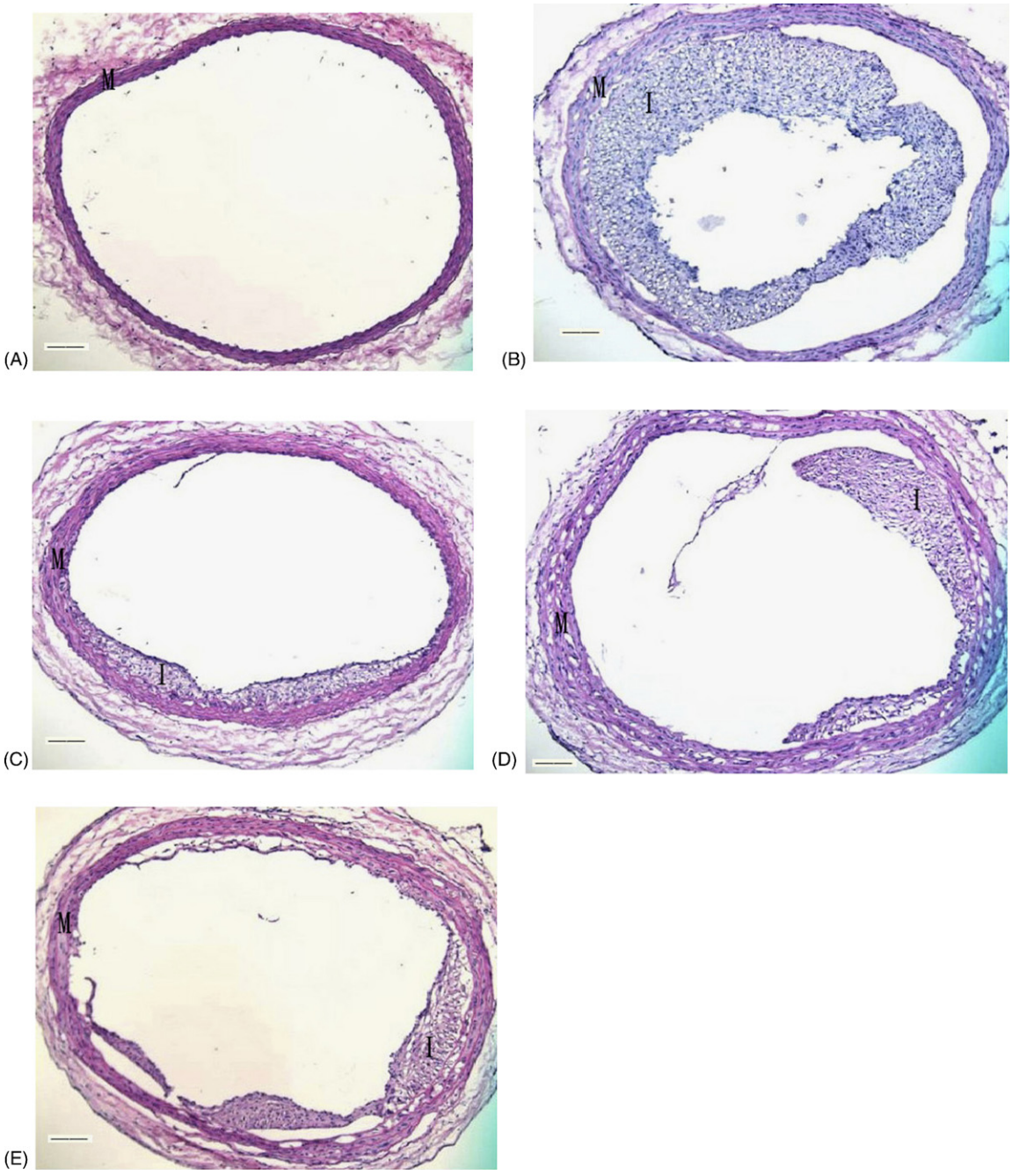

Fig. 4. Representative sections of H&E-stained carotid arteries with or without balloon injury. Non-injured carotid artery (A), arteries treated by normal saline (B), arteries treated by resveratrol in a tocol emulsion (C), arteries treated by resveratrol in a lipid emulsion (D), and arteries treated by resveratrol in an aqueous micelle system (E) (bar scale: 20 μm). I, neointima; M, media.

have surpassed vitamin E's antioxidant capacity (Schneider, 2005). The scavenging activity due to vitamin E is so strong that the further enhancement on scavenging due to resveratrol was limited. Although vitamin E exhibited a higher level of in vitro scavenging activity, this effect did not produce in vivo efficacy on intima decrement. This may have been due to the more-complex condition of the in vivo compared to the in vitro situation. Many factors in the in vivo situation may have affected the final outcome of the drug in the vehicles. Vitamin E may synergize the activity of resveratrol in specific conditions but did not do so in the present case.

3.5. Erythrocyte hemolysis

The use of emulsions for parenteral administration imposes rigorous demands on the non-toxicity of the formulation. To evaluate the safety of the emulsions themselves, the hemolytic activity was determined. The hemolytic potential of the injectable forms has generally been found to correlate with the severity of lesions (Bjerregaard et al., 2001). The hemolysis percentages (%) of the tocol emulsion, lipid emulsion, and aqueous micelle system were -0.69 ± 2.40, 1.21 ± 2.41, and $9.18 \pm 5.55\%$, respectively. Any toxic effect was expected to be

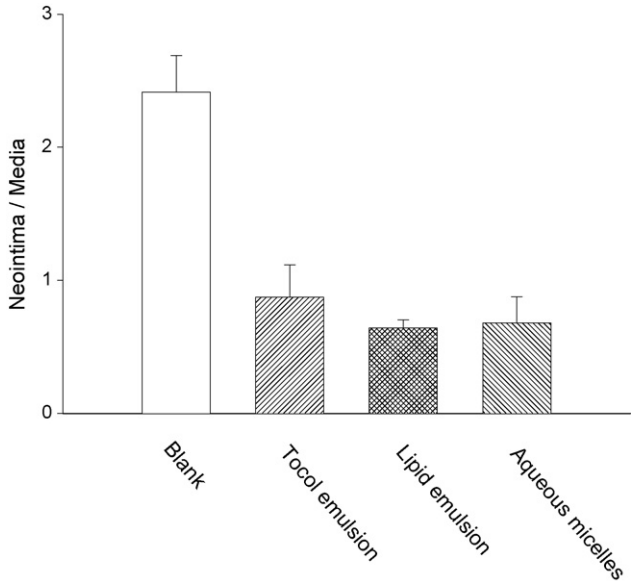

Fig. 5. Inhibition of intimal thickening in the rat injury model treated by normal saline (blank), resveratrol in a tocol emulsion, resveratrol in a lipid emulsion, resveratrol in an aqueous micelle system, as determined by the neointimal/media ratio. Each value represents the mean and S.D. ($n = 3$).

mediated by direct contact between the vesicles and erythrocytes. Phospholipids are known to cause erythrocyte hemolysis (Ishii and Nagasaka, 2004). As suggested previously, phosphatidylcholine used for emulsion preparation is found in two different states: one stabilizes the oil droplets, another disperses in the water phase as liposomal vesicles. The phospholipids in liposomes may easily penetrate through erythrocytes because this vesicle system is more unstable than the oil droplet (Ishii and Nagasaka, 2004). Hence the emulsions are more applicable as compared to liposome based on toxicity considerations. Another mechanism is that the large molecules of PEP and CP sterically shield the lytic effect of phosphatidylcholine. The same phenomenon is seen in lipid emulsions incorporated with triglycerol polyricinoleate-6 (Bjerregaard et al., 2001). Vitamin E may allow for incorporation in the emulsion without increasing toxicity.

3.6. Stability test

Physical stability of an emulsion is one of the most important desired product characteristics. Emulsions are heterogeneous systems and thermodynamically unstable and, therefore, have a significant tendency to lose physical stability during storage. However, the plain emulsion greatly destabilized with 5- and 7-fold increase in vesicle size after 6- and 12-h incubation (Fig. 7). The insufficient concentration of phospholipids for completely emulsifying the emulsion system may be the predominant reason for this result (Brime et al., 2002). The addition of glycerol formal may also destabilize the whole system of plain tocol emulsion. This low stability of plain emulsion may result in the fast release of resveratrol as shown in Fig. 3A. The addition of Brij 35 stabilized the tocol emulsion. The stability of the Brij 35-containing emulsion was correlated with a decrease in droplet size because the size stability of the emulsions depends on the initial particle size (Chung et al., 2001). The further incorporation of PEG derivatives did not influence the stability of the tocol emulsion, although there was a slight but significant increase ($p < 0.05$) in particle size at 12 h (Fig. 7). The surface conju-

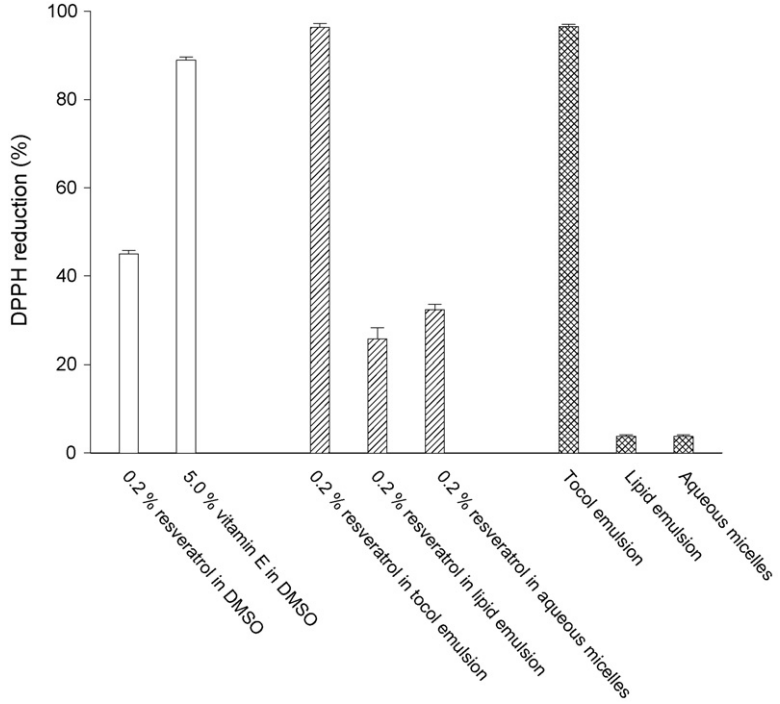

Fig. 6. The DPPH reduction (%) in the in vitro scavenging activity of 0.2% (v/w) resveratrol and 5.0% (w/v) vitamin E formulated in DMSO, tocol emulsion, lipid emulsion, and aqueous micelle system. Each value represents the mean and S.D. ($n = 3$).

Table 3
Droplet size and polydispersity change ratio[a] (fold) of formulations with CP and PEP incubated at 37 °C during 30 days

Formulation	14th day		30th day	
	Size	Polydispersity	Size	Polydispersity
Tocol emulsion	0.97 ± 0.03	0.96 ± 0.01	0.96 ± 0.01	0.96 ± 0.03
Lipid emulsion	1.07 ± 0.03	0.87 ± 0.15	10.75 ± 3.68	0.97 ± 0.11
Aqueous micelles	0.85 ± 0.03	1.15 ± 0.10	16.28 ± 16.96	4.30 ± 1.20

Each value represents the mean ± S.D. ($n = 3$).
[a] The droplet size change ratio = size at 14th day or 30th day/size at 0th day.

gation of PEG increases the hydrophilicity of the vesicles, and lowers their surface free energy (Mao et al., 2001). However, this effect could not prevent the droplet aggregation of the lipid emulsion and liposome system in normal saline (Fig. 7). The aqueous micelle system showed a greater increase in the ratio of mean size as compared to the emulsions. This suggests the instability of the phospholipid bilayers rather than the oil/water interface. The existence of oil components is beneficial to the stability of this system.

The formulations with Brij 35 and PEG derivatives were further incubated at 37 °C for long-term storage. As shown in Table 3, the particle size was almost unchanged in the first 14 days. The liposomal size was even reduced to a size change ratio of 0.85. This was likely caused by particle destruction which is commonly observed during storage (Yamaguchi, 1996). Eleven- and 16-fold increases in particle size were, respectively, observed for the lipid emulsion and aqueous micelle system after a 30-day storage, accompanied by visible deterioration (Table 3). The results showed a growing standard deviation of the size change ratio for liposome. This indicates rising instability as well (Buszello et al., 2000). A similar trend was shown in the polydispersity index (Table 3). No or negligible change in polydispersity was observed for tocol emulsion. However, a two- to four-fold increase in the polydispersity was shown for lipid emulsion and aqueous micelle formulations. This may confirm the instability of these two systems. The use of vitamin E as an antioxidant in emulsions could provide considerable stability for practical use. On visual inspection, all formulations were still homogenous and no visible free oil or breakage of the systems was seen during the long-term stability test. Hence the visual inspection was not enough to judge the emulsion stability.

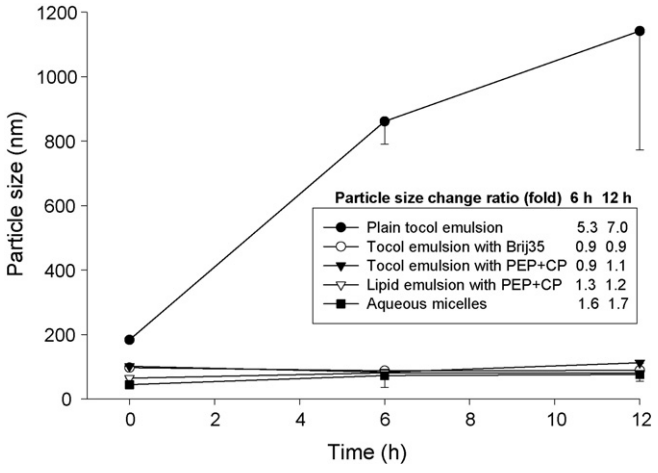

Fig. 7. The change of droplet size of emulsions and aqueous micelles as a function of time incubated in normal saline at 37 °C during 12 h. Each value represents the mean and S.D. ($n = 3$). The particle size change ratio (fold) after 6 and 12 h is shown in the legends.

4. Conclusions

Due to the high lipophilic drug loading, emulsions can be clinically administered undiluted at a high dose without drug precipitation. Injectable emulsions of resveratrol with Brij 35 and PEG derivatives have been developed with a mean size of <100 nm. The system formed without oils (aqueous micelles) had further reduced vesicle sizes. Contrary to the aqueous micelle system, the incorporation of vitamin E (5%) in the emulsion (tocol emulsion) resulted in enlarged vesicle sizes. Compared to the in vitro resveratrol release from the emulsions, drug release from the micelle system was faster. The in vivo results showed that resveratrol limited neointimal hyperplasia following arterial injury in rats. Although vitamin E incorporation increased the in vitro scavenging activity, no significant difference was observed in the degree of hyperplasia inhibition by the tocol emulsion, lipid emulsion, or aqueous micelle system. The results of erythrocyte hemolysis suggested that the incorporation of oil (tocol and lipid emulsions) reduced the acute toxicity by phospholipids. The physical stability of the emulsions, especially the tocol emulsion, was greater than that of the aqueous micelle system. Emulsification of the drug into an oil/water system can be an important approach towards an aqueous formulation system. The incorporation of vitamin E in the system should be deliberated based on the concerns of safety, storage stability, and therapeutic efficacy.

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