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**ORIGINAL ARTICLE**

# The composition of oil phase modulates venous irritation of lipid emulsion-loaded diallyl trisulfide

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**Abstract**

*Introduction*: In this study, a nanoemulsion system (LE) was investigated for intravenous delivery of diallyl trisulfide (DT), which was a lipophilic and venous irritant drug for systemic therapy of bacterial and fungal infection. *Methods*: Egg phospholipid was chosen as the only emulsifier, soybean oil, medium chain triglyceride (MCT), and olive oil were used as the oil phases, forming stable DT LEs (o/w) with small particle sizes. The venous irritation of DT LEs was evaluated by in vitro human umbilical cord endothelial cells (HUV-EC CRL 1730) tolerance model with the intracellular ATP and GTP concentrations as the indices. *Results*: The intracellular ATP and GTP reduction changed with the incorporation of a variety of oils, which were strongly related with the free DT concentration of DT LEs. *Discussion*: It was deduced that the free DT concentrations of LEs made of various oils depended on the particle sizes of the DT LEs. In conclusion, the oil phases modulated the free DT concentrations by forming DT LEs with different particle sizes, and optimization of the oil phase was an effective method to alleviate the venous irritation of DT LEs.

**Key words:** *Diallyl trisulfide; lipid emulsion; oil phase; particle sizes; venous irritation*

## Introduction

Venous irritation was the most common local complication and a major problem in intravenous delivery of venous irritant drugs[1–3], such as propofol[4] and clarithromycin[5]. Then, lipid emulsion (LE) was developed as parenteral drug delivery system because of the significant advantages including reducing injection pain and venous irritation[1,6,7]. Nonetheless, about half of patients still experienced moderate venous irritation and pain on injection when the propofol emulsion (Propofol-Lipuro™) was administrated. On the contrary, the drug-loaded emulsion made of medium-chain triglycerides (MCTs) could produce less pain and venous irritation compared with that made of long-chain triglycerides, such as soybean oil[8–10]. This suggested that the composition of oil phase had great effect on the venous irritation of emulsions loaded with lipophilic and venous irritant drugs.

In China, diallyl trisulfide (DT) is a lipophilic organosulfur compound from garlic (*Allium Sativum*), and it is commercially available as a preparation, known as Dasuansu, which is prescribed for the treatment of bacterial infections[11–13] and systemic fungal infections[14]. The current commercial formulation of DT (DT IV Solution) employs DT in a Tween 80 and propylene glycol solution, but this formulation has annoying side-effect, such as venous irritation and occasional thrombophlebitis[15]. Hence, to overcome these problems and satisfy patient compliance, LE appears to be a viable alternative for the intravenous administration of DT. In our preliminary experiments with rabbits, when DT was loaded in the standard emulsion (10% Intralipid®), the venous irritation symptom of DT LE was milder than that of DT solution. However, the result did not satisfy our requirement.

The aim of this work is to study the effects of the composition of oil phase on the venous irritation of LEs for intravenous delivery of DT with extremely lipophilic and serious venous irritant characteristics. Although venous irritation is the most common side-effect of intravenous therapy, its pathogenesis is not fully understood, the most prevalent opinion being that chemical irritation of the endothelium leads to a sterile inflammation[1,16]. Moreover, many studies disclosed that the venous irritation of drug-loaded LE should be

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attributed to the free drug in the aqueous phase[17–19]. Thus, we suspected that modulation of the free DT concentration by the oil phase was an efficient method of reducing the venous irritation of parenteral DT LE, and the hypothesis was verified in this article.

## Materials and methods

### *Materials*

DT was provided by Qingjiang Pharmaceutical Corp. (Huaian, China). Soybean oil was purchased from Tieling Beiya Pharmaceutical Corp. (Tieling, China). Egg lecithin (Lipoid E 80®), oleic acid, and olive oil were provided by Shanghai Dongshang Corp. (Shanghai, China). MCT was kindly donated by BASF Co., Ltd. (Shanghai, China). Glycerol, propylene glycol, and Tween 80 were obtained from Shanghai Chemical Reagent Corporation (Shanghai, China). Other chemicals are of high-performance liquid chromatography (HPLC) or analytical grade. Double-distilled water was used for all preparations.

### *Preparation of DT lipid emulsion*

The LEs used in this study were as follows: oil (10%, w/v), egg lecithin (1.2%, w/v), oleic acid (0.03%, w/v), glycerol (2.21%, w/v), and DT (1.5%, w/v), when expressed as a percentage of the total volume of the emulsions. The LEs were prepared by a standard method[20]. Briefly, the oil phase containing DT, oleic acid, and egg lecithin was mixed with the aqueous phase containing glycerol at 50°C; the primary emulsion was sheared using a high-shear mixer (Model 1001; Shanghai Weiyu Corp., Shanghai, China) at 8000 rpm for 15 minutes and homogenized with a high-pressure homogenizer (APV 2000, Invensys plc, London, UK) at four cycles and 1000 bar. Subsequently, the pH values of the LEs were adjusted to 7.00 ± 0.05 using 0.1 M sodium hydroxide solution.

To get control DT IV solution, stock solution containing 1.50 g DT was prepared with the mixture of 9 g Tween 80 and 10 g propylene glycol; next, the stock solution was added to the water drop by drop under a magnetic stirring at ambient temperature, and then the solution was diluted to 100 mL.

The final DT LE and IV solutions were packaged in 20-mL ampoules, sealed after nitrogen purging, and then sterilized by autoclaving (121°C, 20 minutes).

### *Vesicle size and zeta potential*

The mean particle size and zeta potential of DT LEs were determined by photon correlation spectroscopy (Nano ZS 90; Malvern Instruments, Worcestershire, UK). DT LEs were diluted 40 times with double-distilled water before measurement and three samples were prepared for one LE.

### *Stability of lipid emulsions*

The DT LEs were stored for 3 months at 4°C and assayed for physical stability. The mean particle size and zeta potential were used as indicators of physical stability.

### *Endothelial cell tolerance of DT lipid emulsions*

#### Cell cultures

Human umbilical cord endothelial cells (HUV-EC CRL 1730, ATCC) at passage 12 were grown following the standard protocol[21]. The cells for experiments were cultured at a density of $2 \times 10^4$ cells/cm$^2$ in 35 mm diameter dishes.

#### Incubation with lipid emulsions

Confluent cells for irritation tests were obtained after 4 days and counted using a hemocytometer. Test solutions were prepared by diluting the samples, 1 mL in 4 mL with phosphate-buffered saline (PBS). The medium was discarded from confluent cells, and the cells were washed twice with PBS (pH 7.4), then test solutions (1 mL) were added, and cells were incubated for 30 minutes at 37°C. Control cells were exposed to PBS alone. Next, the cells were washed twice with PBS after test and control solutions were removed. Thereafter cells were lysed by the addition of 150 µL cold 0.42 M perchloric acid solution and stored at −20°C for 30 minutes. Finally, the cell lysate was neutralized with 150 µL cold 1 M potassium phosphate dibasic solution (pH > 7.2). After centrifugation, 250 µL supernatant was collected and immediately frozen and stored at −20°C until chromatographic analysis.

#### Determination of intracellular adenosine triphosphate and guanosine triphosphate via HPLC

Separation and quantification of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) in the perchloric acid extracts were made by reversed-phase HPLC as previously described[22]. The concentrations of ATP and GTP were quantified by determining the ratio of peak areas in relation to corresponding standards. The linear range for all two nucleotides was between 0.75 and 50 µM. Then the percentage of intracellular ATP and GTP reduction was calculated for each formulation by the following equation:

$$\%\ (\text{ATP or GTP reduction}) = \frac{C_{\text{control}} - C}{C_{\text{control}}} \times 100,$$

where $C$ is the ATP or GTP concentration of cells incubated with the formulation and $C_{\text{control}}$ is the ATP or GTP concentration of cells incubated with PBS control.

### *Determination of the DT concentration in the aqueous phase of lipid emulsions*

The DT concentrations in the aqueous phase of LEs were determined through reverse dialysis bag technique[18,23,24]. Test solutions were prepared by diluting the DT LEs 1 mL in 4 mL with PBS. Test solutions (100 mL) were placed in the Erlenmeyer flask (150 mL) with magnetic stirring; three dialysis bags (MWCO 3500; Biotech, Wuhan, China) containing 1 mL of 2.5% glycerol solution were immersed. These sacs were equilibrated with the glycerol solution for 12 hours prior to experiment. After equilibration for 24 hours at ambient temperature, the dialysis bags were withdrawn and the concentration of DT in the contents was assayed by HPLC (Model 1100; Agilent, Santa Clara, CA, USA), equipped with a UV detector set at 240 nm and a reversed-phase column (Elite-$C_{18}$, 5 μm, 4.6 mm ID × 25 cm; Dalian, China). The mobile phase was a mixture of water and methanol (20:80, v/v) and the flow rate was 1.0 mL/min. Preliminary experiments were conducted to determine the time to reach equilibrium. Samples were collected and analyzed at 3, 6, 12, 24, 36, and 48 hours. It showed that the equilibrium was achieved within 24 hours. Each sample was measured in triplicate.

### *Determination of partition coefficients between oil and water of DT*

Twenty milliliters of oil containing DT was kept in contact with 20 mL of pH 7.0 PBS in a 100 mL vial at 25°C for 48 hours. Preliminary experiments were conducted to determine the time to reach equilibrium. Samples were collected and analyzed at 24, 48, 72, and 96 hours; the results showed that equilibrium was achieved within 48 hours. After equilibrium, the two phases were separated by centrifugation and the aqueous samples were assayed using HPLC. These experiments were repeated three times.

### *Data analysis*

Statistical analysis of differences between different treatments was performed using analysis of variance (ANOVA) test, using SPSS 12.0 for windows (SPSS software; LEAD Technologies, Haddonfield, NJ, USA). $P < 0.05$ was considered to be statistically significant.

## Results and discussion

### *Physicochemical characteristics*

The particle sizes of the DT LEs made of various oil phases are presented in Table 1. The particle size of DT LE made of olive oil is the smallest, followed by soybean oil DT LE, and then MCT DT LE. The difference of the particle sizes may be attributed to the fatty acid composition of the oil. The typical fatty acid composition of soybean oil and olive oil is mainly unsaturated fatty acids, which are oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), and a small part of saturated fatty acids including palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$). But that from MCT are saturated fatty acids like octanoic acid ($C_{8:0}$), decanoic acid ($C_{10:0}$), and lauric acid ($C_{12:0}$)[25]. This may indicate that medium-chain saturated fatty acids produce larger droplets than long-chain unsaturated fatty acids[26]. On the other hand, the particle size of LEs is also related to the viscosity of the oil phase; when the viscosities were lower than a threshold value of 100 mPa s, the particle sizes of LEs gradually decreased with increasing viscosities, whereas they were not affected by the surfactant concentration[27]. The results in the study meet the theory well because the viscosities of the three oils (olive oil, soybean oil, and MCT) were all lower than 100 mPa s at LE preparation temperature (50°C) and they were in the decreased order exactly[28].

**Table 1.** The particle size and zeta potential characterization of DT lipid emulsions.

| Lipid emulsions | Size (nm) | Zeta potential (mV) | Polydispersity index |
|---|---|---|---|
| Soybean oil (10%) blank LE | 245.7 ± 7.4 | −63.2 ± 2.3 | 0.07 |
| Soybean oil (10%) DT LE | 248.5 ± 8.3 | −64.9 ± 3.1 | 0.06 |
| MCT (10%) DT LE | 317.2 ± 7.9 | −65.2 ± 0.8 | 0.03 |
| Olive oil (10%) DT LE | 187.7 ± 6.9 | −64.1 ± 2.1 | 0.12 |
| Soybean oil (5%) + MCT (5%) DT LE | 284.6 ± 6.9 | −63.9 ± 2.5 | 0.08 |
| Soybean oil (5%) + olive oil (5%) DT LE | 215.8 ± 4.3 | −65.7 ± 0.9 | 0.09 |

Each value represents the means of three measurements ± SD.

### *Stability of lipid emulsions*

All DT LE systems were physically stable at 4°C during the 3 months. Phase separation had not been observed during the period.

The solubility of the oils in the water has a major impact on physical stability and the insolubility of triglyceride oils in water acted as a kinetic barrier to Ostwald ripening, making DT LEs made of the three oils inherently stable to Ostwald ripening[29]. Another reason might be the addition of 0.03% oleic acid, which increased the negative zeta potential, leading to improve the stability of DT LEs[30].

### *Endothelial cell tolerance of DT lipid emulsions*

There were a series of methods to evaluate the venous irritation of parenteral drug formulations at the injection site, such as in vivo rabbit ear model, in vitro cell tolerance model, and in vitro hemolysis test[3,31]. The in vitro cell tolerance model (HUV-EC CRL 1730, etc.) was appreciated for its accurate assessment and no perplexity of animal ethics[21,32,33]. By determining the intracellular contents of ATP and GTP of the treated cells, intact endothelial metabolism was examined[32–34].

As the results shown in Figure 1, intracellular ATP and GTP reduction of the cells treated by the blank LEs are all slight, and there is no significant difference among the blank LEs made of three oils and mixture; they are safe in clinical use[35,36].

The effects of DT solution and DT LEs made of three oils and their mixtures on intracellular ATP and GTP levels are presented in Figure 2. One-way ANOVA shows that soybean oil DT LE and DT solution significantly reduce the intracellular ATP and GTP ($P < 0.01$) compared with the blank soybean oil LE. It demonstrates that the intracellular ATP and GTP reduction in the endothelial cells are attributed to the cytotoxicity nature of DT. The report that 50 μg/mL DT in the medium might adversely affect the viability of the hepatocytes also corroborates the venous irritant nature of DT[37]. On the other hand, the ATP and GTP reduction treated with the DT solution are significantly higher than that of the soybean oil DT LEs ($P < 0.01$). This suggests that DT is responsible for the endothelial cell damage, and encapsulation of DT in LE could shield the endothelial cells from the cytotoxicity of DT[38].

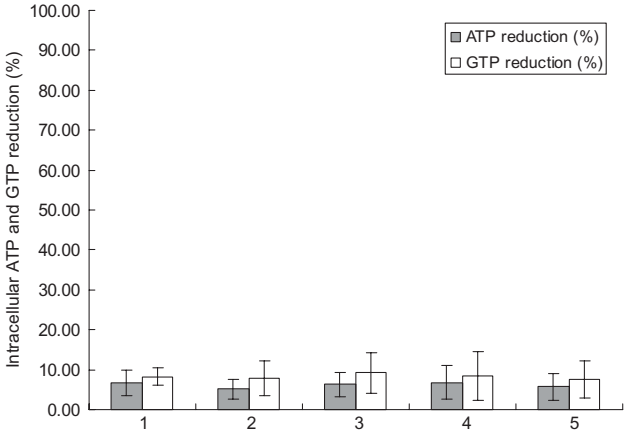


**Figure 1.** Effect of the lipid emulsions with various oil phases on intracellular ATP and GTP reduction (%) in HUV-EC CRL-1730 cell line. (1) Soybean oil (5%) blank LE, (2) soybean oil (5%) + olive oil (5%) blank LE, (3) olive oil (10%) blank LE, (4) soybean oil (5%) + MCT (5%) blank LE, (5) MCT (10%) blank LE. Each value represents the means of three measurements ± SD.

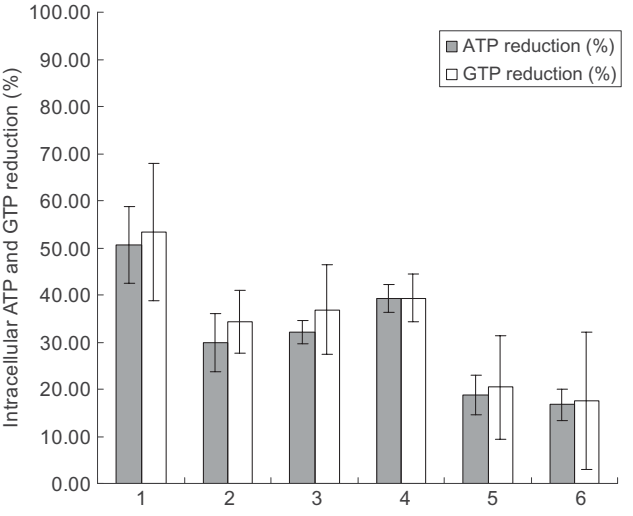


**Figure 2.** Effect of the DT lipid emulsions with various oil phases on intracellular ATP and GTP reduction (%) in HUV-EC CRL-1730 cell line. (1) DT IV solution, (2) soybean oil (10%) DT LE, (3) soybean oil (5%) + olive oil (5%) DT LE, (4) olive oil (10%) DT LE, (5) soybean oil (5%) + MCT (5%) DT LE, (6) MCT (10%) DT LE. Each value represents the means of three measurements ± SD.

Post-Dunnett's tests following ANOVA show that intracellular ATP and GTP reduction of cells exposed to the olive oil DT LEs were significantly higher than that of the soybean oil DT LE ($P < 0.05$), and the intracellular ATP and GTP reduction aggravates with the increasing olive oil percentage in the LE. Conversely, compared with the soybean oil DT LE, the intracellular ATP and GTP reduction of cells exposed to the MCT DT LEs were significantly lower ($P < 0.01$). Moreover, the DT LEs with higher percentage of MCT bring less reduction of the intracellular ATP and GTP ($P < 0.01$). This indicates that DT LEs made of MCT have better compatibility with the endothelial cells. The results coincide well with the report that LCT/MCT propofol reduced injection pain compared with the LCT propofol[4,8,17]. Many studies disclosed that the venous irritation of drug-loaded LE should be attributed to the free drug concentration of aqueous phase[17–19]; we also hypothesized that the free DT concentration of aqueous phase may be the decisive factor of the venous irritation frequently associated with the DT LEs.

### *Modulation of the DT concentration in the aqueous phase of lipid emulsions*

Figure 3 presents the results of the DT concentrations in the aqueous phase of LEs made of different oils. The DT concentration in the aqueous phase of DT LE made of olive oil is the highest, followed by soybean oil, and then MCT.

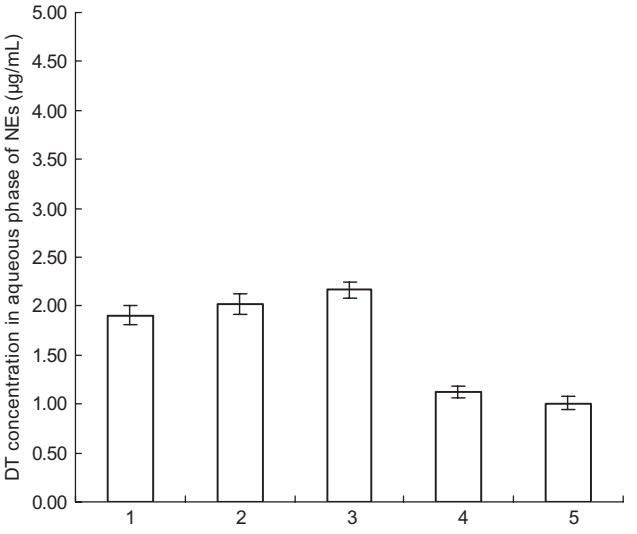


**Figure 3.** Effect of the oil phase composition on the DT concentration in the aqueous phase of the DT LEs. (1) Soybean oil (10%) DT LE, (2) soybean oil (5%) + olive oil (5%) DT LE, (3) olive oil (10%) DT LE, (4) soybean oil (5%) + MCT (5%) DT LE, (5) MCT (10%) DT LE. Each value represents the means of three measurements ± SD.

We investigated the effects of the oil phase on the DT concentrations in the aqueous phase of LEs. The theoretical mechanism of drug interfacial transport based on Fick's first law of diffusion is applied to predict the mass transport phenomena at an interfacial membrane of o/w LE in vitro. Hosokawa et al.[39] deduced the percentage of the drug in the aqueous phase of o/w emulsion, *Qa* (%), which is given by Equation (1):

$$\%Qa = 100 - 100 \times \exp\left(-\frac{6P}{d} \times t\right), \quad (1)$$

where *P* is the apparent permeability of drug across the interfacial membrane and *t* is the unit time. *d* is the mean particle size, which was measured by dynamic light scattering.

The *Qa* can be expressed as ($Ca \times Va$), *Ca* is the drug concentration in the aqueous phase, and *Va* is the volume of aqueous phase. Furthermore, the permeability *P* is given by

$$P = \frac{Ds}{Kds \cdot \Delta s} \quad (2)$$

where *Kds* is the solute (drug) partition coefficient between the dispersed phase (oil) and the continuous phases (water), *Ds* is the effective diffusion coefficient through the surfactant layer (interfacial membrane), and $\Delta s$ is the surfactant layer thickness[40]. Hence, rearrangement of Equation (1) yields

$$\%(Ca \times Va) = 100 - 100 \times \exp\left(-\frac{6Ds}{Kds \cdot \Delta s \cdot d} \times t\right) \quad (3)$$

Considering Equation (3), the DT concentrations in the aqueous phase of DT LEs, *Ca*, are determined by the four parameters: *Kds*, *Ds*, $\Delta s$, and *d*. As far as the DT LEs are concerned, the interfacial membranes are all made of the phospholipids and the amounts of the phospholipids in the formulations are the same. Thus, it is speculated that the parameters of $\Delta s$ are almost the same. In addition, the diffusion parameters *Ds* of the DT LEs are identical, because the initial concentrations of DT in the disperse phases are same. Thus, the product of ($Kds \times d$) is the critical factor in the determination of the DT concentration of the aqueous phase.

Combining the results of particle sizes with the values of logarithm of partition coefficients showed in the Tables 1 and 2, the products of ($Kds \times d$) of the DT LEs made of olive oil, soybean oil, and MCT are in increasing order. Accordingly, the calculated results of *Ca* of DT LEs are in decreasing order. It is emphasized that the deduced results are well in accordance with the experimental results that are presented in the Figure 3.

On the other hand, it is found that there is strong relationship between the results of particle sizes and the free DT concentration of DT LEs. In other words, the DT LEs with smaller particle size have higher DT concentrations in the aqueous phase. Based on Equation (2), we can predict that the drug concentration of aqueous phase will be higher in the LE with smaller particle size; the experimental results agree well with this corollary. LEs made of different oils showed different particle size, which could influence the in vitro release properties of lipohilic drugs and the concentration of drug in the aqueous phase[41]. The drug in smaller particles has higher chemical potential than that in larger particles, because of the increased internal pressure due to the highly curved particle surface[39]. Hence, it is concluded that the DT LEs made of various oils regulate the free

**Table 2.** The logarithm of oil/water partition coefficients of DT in 0.05 M phosphate buffer (ionic strength = 0.2) at 25°C.

| Oil | Logarithm of partition coefficients |
|---|---|
| Soybean oil | 3.87 ± 0.13 |
| MCT | 3.57 ± 0.09 |
| Olive oil | 4.03 ± 0.17 |

The data are means of three measurements ± SD.

DT concentrations through forming particles with different sizes.

## Conclusion

The DT LEs for intravenous delivery were prepared and evaluated using in vitro cell model. Various oils were selected to prepare stable LEs for investigating the effects of oil phase on the venous irritation of DT LEs. The results showed that the oil phase composition could regulate the venous irritation of the DT LEs. In addition, the DT LE made of MCT showed the best cell compatibility. The incidence of HUV-EC compatibility varied in a free DT concentration-dependent manner. Moreover, the oils regulated the free DT concentrations through forming particles with different sizes. In conclusion, LE was an appropriate delivery system for lipophilic and venous irritant drugs, and optimization of the formulation was an effective method to alleviate the venous irritation of DT LEs.

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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