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# Influence of vitamin E tocopherol polyethylene glycol succinate 1000 on intestinal absorption of icariside II

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In the present study, the Caco-2 monolayer model and a four-site rat intestinal perfusion model were used to investigate the effects of vitamin E tocopherol polyethylene glycol succinate 1000 (TPGS) on the intestinal absorption of icariside II. Icariside II was analyzed by ultra-performance liquid chromatography (UPLC). After that its apparent permeability coefficients (Papp) and effective permeability ($P^*_{eff}$) were calculated. In the Caco-2 cell model, Papp values from the apical (AP) to the basolateral (BL) of icariside II were increased and its efflux ratios were markedly reduced in the presence of TPGS. However, either 0.25 mg/mL or 0.5 mg/mL of TPGS had no significant difference in promoting the absorption of icariside II. In four-site rat intestinal perfusion model, $P^*_{eff}$ of icariside II were significantly increased by 0.5 mg/mL of TPGS in ileum and colon. The results suggest that TPGS could promote the intestinal absorption of icariside II.

## 1. Introduction

Herba Epimedii (Berberidaceae), also named YinYang Huo, is commonly used in traditional Chinese medicine for the treatment of sexual dysfunction, rheumatism, osteoporosis, hypertension, coronary heart disease, bronchitis, chronic hepatitis and viral myocarditis (Lin et al. 2004; Liu et al. 2005). The major active constituents of Herba Epimedii are prenylated (specifically isopentenyl) flavonoids, including icariin, epimedin A, epimedin B, epimedin C and icariside II etc. (Yap et al. 2005; Guo and Xiao 2003; Zhang et al. 2008). Icariside II (Fig. 1), a metabolite of icariin, is a prenylated flavonoid glycoside with the chemical structure of $C_{27}H_{30}O_{10}$ and a molecular weight of 514 Da. Its varieties of biological activities have also been reported (Guo and Xiao 2003). Icariside II has shown to have cytotoxic and cytostatic effects on 6 kinds of cancer cell-lines by inhibiting the synthesis of DNA and RNA (Li et al. 1990). It has been found that icariside II could enhance the differentiation and proliferation of osteoblasts, facilitate matrix calcification, and inhibit osteoclastic differentiation in both osteoblast–preosteoclast coculture and osteoclast progenitor cell culture, as well as reduce the motility and bone resorption activity of isolated osteoclasts (Huang et al. 2007). Meanwhile it has been reported that icariside II could inhibit lymphocyte activation and suppress T-cell activation in T cell receptor/CD3-mediated signaling pathways in a dose- and time-dependent manner (Ma et al. 2004, 2005). Icariside II might be developed into new drugs against anticancer, osteoporosis and immunity-related diseases. However, it has low absorptive permeability for apical efflux via breast cancer resistance protein (BCRP), multidrug resistance-associated protein 1 and 2 (MRP 1, MRP 2) and P-glycoprotein (P-gp) (Chen et al. 2008).

Vitamin E tocopherol polyethylene glycol succinate 1000 (TPGS) has a hydrophilic (PEG) head and a lipophilic (phytyl) tail, has been used as the solubilizer, emulsifier and as vehicle for lipid-based drug delivery formulations. Most recently, TPGS 1000 has been recognized as an effective oral absorption enhancer by surfactant-induced inhibition of P-glycoprotein (P-gp) and perhaps other drug transporter proteins (Bhagwant et al. 2002; Collnot et al. 2006; Wempe et al. 2009).

Here, the effect of TPGS on the intestinal absorption of icariside II were investigated by using the Caco-2 monolayers cell model and the four-site rat intestinal perfusion model, which were recognized by the FDA as viable models of human intestinal absorption (Chen et al. 2008; Blume et al. 2010; Degim et al. 2010).

## 2. Investigations, results and discussion

### *2.1. Effect of icariside II and TPGS on transepithelial electrical resistance (TEER)*

Before investigating the effects of TPGS on the transport of icariside II across a Caco-2 cell monolayer, it was important to determine whether icariside II and TPGS affects cell monolayer integrity. In this study, TEER measurements were used to test the cellular integrity in the presence of 20 μM icariside II or 0.25 mg/mL TPGS or 0.5 mg/mL TPGS over a period of 4 h. Changes of icariside II and different concentration of TPGS groups in TEER were not considered significant ($p > 0.05$) compared to controls, which indicated icariside II and TPGS have no damage to the cell monolayers.

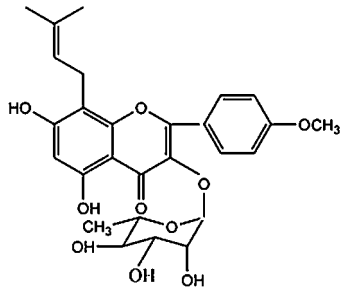


Fig. 1: Chemical structure of icariside II

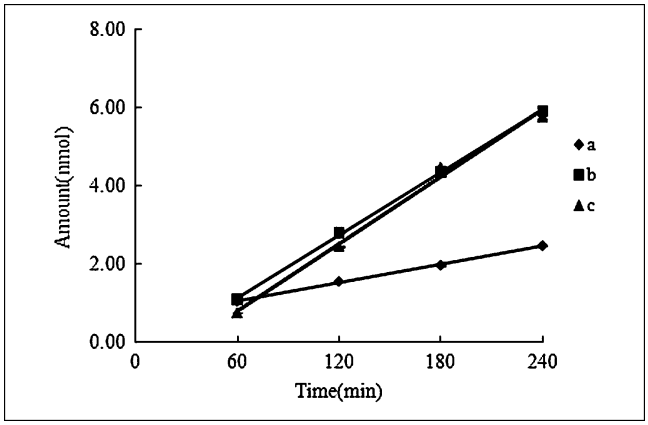


Fig. 2: Transportation of icariside II in different concentrations of TPGS from apical to basolateral during 4 h (a: 20 μM icariside II, b: 20 μM icariside II with 0.25 mg/mL TPGS, c: 20 μM icariside II with 0.5 mg/mL TPGS). Data presented as mean ± S.D.; each experimental group represents studies across three transwells (n = 3)

### *2.2. The influence of TPGS on icariside II in Caco-2 cell monolayer*

The flux of icariside II (20 μM) across Caco-2 cellmonolayers in the absorptive (AP–BL) and in the secretory (BL–AP) directions and the corresponding Papp values, in the absence or presence of TPGS, is shown in Table, Fig. 2 and Fig. 3. It can be seen that icariside II displayed a polarized transport, i.e., significantly higher Papp value in the BL–AP in comparison to the AP–BL direction, with an efflux ratio ($Papp_{BL–AP}/Papp_{AP–BL}$) of 15.3. In the presence of 0.25 mg/mL TPGS, the absorptive permeability ($Papp_{AP–BL}$) of icariside II was increased by 3.4 times and the secretory permeability ($Papp_{BL–AP}$) was reduced by 47%, which results a reduction of 84% in the efflux ratio. On the other hand,

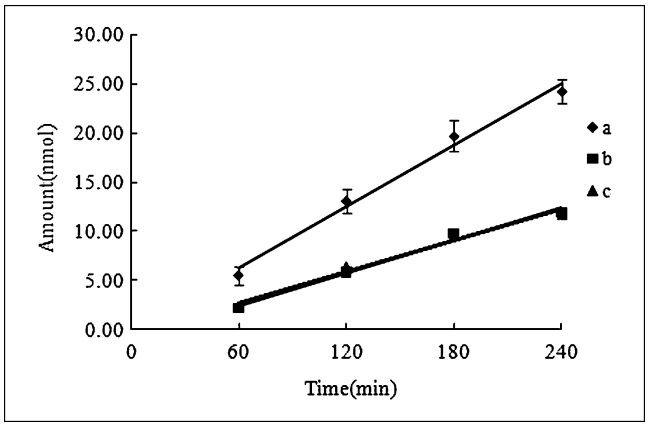


Fig. 3: Transport of icariside II in different concentration of TPGS from basolateral to apical during 4 h (a: 20 μM icariside II, b: 20 μM icariside II with 0.25 mg/mL TPGS, c: 20 μM icariside II with 0.5 mg/mL TPGS). Data presented as mean ± S.D.; each experimental group represents studies across three transwells (n = 3)

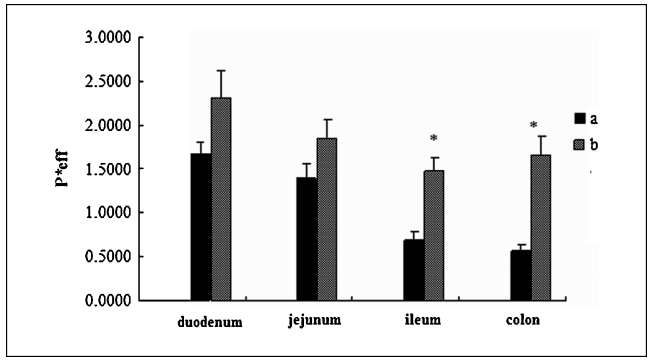


Fig. 4: Comparison of permeability of icariside II in different intestinal segment in the presence or absence of TPGS (a: 20 μM icariside II, b: 20 μM icariside II with 0.5 mg/mL TPGS). Data presented as mean ± S.D.; n = 3; *p < 0.05 vs absence of TPGS group

in the presence of 0.5 mg/mL TPGS, neither the absorptive permeability nor the secretory permeability changed significantly ($p > 0.05$) in comparison to 0.25 mg/mL TPGS, which indicates that the concentrations at 0.25 mg/mL and 0.5 mg/mL had the same effect on improving the intestinal absorption of icariside II. The efflux transporter proteins are highly expressed in the intestine and restrict the absorption of its substrates to various extents. Confluent Caco-2 monolayers exhibit morphological and functional similarities to the small intestinal epithelium. They possess efflux transporter proteins such as P-gp, BCRP, MRP1 and MRP2 (Bohets et al. 2001). When icariside II and TPGS were treated together, the secretory permeability of icariside II was decreased significantly. It is indicated that TPGS might inhibit the P-gp, accordingly increased the absorption of icariside II. Those results suggested that icariside II might be the substrate of BCRP, MRP and P-gp, which was supported by the report of Chen et al. (2008).

### *2.3. Influence of TPGS on icariside II in the four-site rat intestinal perfusion model*

The $P_{eff}^{*}$ obtained for icariside II in the presence or the absence of TPGS, in the four-site rat intestinal perfusion, were presented in Fig. 4. Without TPGS, the $P_{eff}^{*}$ of icariside II were $1.657 \pm 0.139$, $1.389 \pm 0.169$, $0.690 \pm 0.095$ and $0.548 \pm 0.070$ in the duodenum, jejunum,ileum and colon, respectively. In the presence of 0.05%TPGS, the $P_{eff}^{*}$ of icariside II was increased significantly in ileum and colon ($p < 0.05$). Following *in situ* perfusion, about 0.07 nmol icariside II was excreted into the bile which got further increased 2∼4 times in the presence of TPGS, shown in Fig. 5. The result suggested that enterohepatic circulation of icariside II might exist.

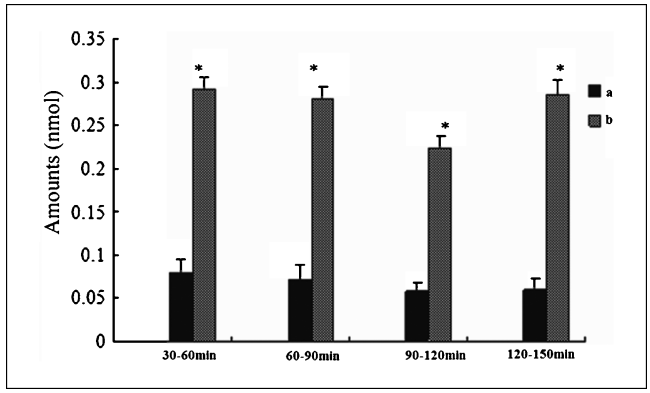


Fig. 5: Comparison of the content of icariside II in bile in the presence or absence of TPGS (a: 20 μM icariside II, b: 20 μM icariside II with 0.5 mg/mL TPGS). Data presented as mean ± S.D.; n = 3; *p < 0.05 vs absence of TPGS group

**Table: Permeabilities (Papp) and efflux ratios ($P_{AP-BL}/P_{BL-AP}$) of Icariside II in the presence or absence of TPGS**

| Samples | Papp($10^{-6}$cm·s$^{-1}$) | | |
|---|---|---|---|
| | AP-BL | BL-AP | $P_{AP-BL}/P_{BL-AP}$ |
| 20 μM icariside II | 1.50 ± 0.016 | 22.98 ± 0.115 | 15.3 |
| 20 μM icariside II + 0.25 mg/mL TPGS | 5.12 ± 0.067* | 12.16 ± 0.243* | 2.38* |
| 20 μM icariside II + 0.5 mg/mL TPGS | 5.47 ± 0.109* | 11.62 ± 0.172* | 2.12* |

Data presented as mean ± S.D.; each experimental group represents studies across three transwells (n = 3); *p < 0.05 vs absence of TPGS group

The four-site rat intestinal perfusion model measures the disappearance of the drug from perfused intestinal segment, directly describing its uptake into the enterocyte (Arik and Amidon 2009). Chen et al. (2011) found that in spite of the presence of a monorhamnose bond at the 3-O position in icariside II, no metabolites were detected after perfused icariside II. The result suggested that the monorhamnose bond could not be hydrolyzed by enzymes in the intestine. Icariside II could be absorbed by enterocytes directly, and the amounts that were absorbed in duodenum and jejunum were much higher than in ileum and colon ($p < 0.05$). Our results were in concordance with those.

The pattern of nonionic surfactant—TPGS had an effect on icariside II permeability obtained in this study, and especially had higher effect in the ileum and colon in comparison to the duodenum and jejunum. This was in corroboration with this pattern that the efflux protein expression follows a gradient pattern, increasing from the proximal regions to the distal small intestinal segments (Gonzalez-Alvarez et al. 2004). Those suggested that the efflux protein inhibition is the main mechanism behind the effects observed in this study. The result showed that the four-site rat intestinal perfusion model was consisted with in Caco-2 cell model.

TPGS has been used as a solubilizer with an excellent safety profile, and the clinical data showed it could also enhance the bioavailability of amprenavir——a marketed antiviral drug (Bhagwant et al. 2002). It was reported that TPGS might logically construct non-ionic surfactants with enhanced propensity to inhibit *in vitro* efflux (Bhagwant et al. 2002; Collnot et al. 2006). All of above indicated that TPGS could enhance the inhibitory potency *in vitro* and *in vivo*. Moreover, our results confirmed that TPGS might inhibit the P-gp and other drug transporter proteins, and increase the intestinal absorption of icariside II. Hence TPGS might be used as the enhancer of icariside II.

In summary, the Caco-2 monolayers cell model and the four-site rat intestinal perfusion model both suggested that TPGS could improve the intestinal absorption of icariside II.

## 3. Experimental

### 3.1. Chemicals

Icariside II (all purity > 98%) was provided by the Laboratory of Pharmaceutical Preparation (Jiangsu Provincial Academy of Chinese Medicine, China). TPGS and Hanks' balanced salt solution (HBSS; powder form) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum was from HyClone (Logan, UT, USA). All other materials (typically analytical grade or better) were used as received.

### 3.2. Cell culture

Caco-2 cells were a kind gift from Dr. Moniqué Rousset of INSERM U178, (Villejuit, France). It was cultured in a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C. The Dulbecco's modified Eagle's medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acid solution, 100 units/ml penicillin, and 100 pg/ml streptomycin. When the cell culture reached 80% confluence, it was rinsed with phosphate-buffered saline and split using trypsin. For transport experiments, the cells were seeded on 3 μm porous polycarbonate cell culture inserts from Nunc, which has a surface area of 4.2 $cm^2$ at a density of 2,500,000 cells/$cm^2$. The culture media was changed every other day. The monolayers were ready for experiments from19 to 22 days after seeding.

### 3.3. Transport experiments in the Caco-2 cell culture model

Experiments were performed in pH 7.4 HBSS. The protocol for performing cell culture experiments was that described previously (Chen et al. 2008). Briefly, the cell monolayers were washed three times with 37 °C HBSS (pH 6.5). The transepithelial electrical resistance (TEER) values of cell monolayers were measured, and those values less than 425 Ω × $cm^2$ (after subtracting the blank) were discarded. The monolayers were incubated with the buffer for 1 h, and the incubation medium was then aspirated. Afterward, a solution containing 20 μM icariside II was loaded on to the apical or basolateral side. The donor samples (400 μL) and the receiver samples (400 μL) were taken at 0, 1, 2, 3, and 4 h after incubation, followed by the addition of 400 μL of fresh donor solution to the donor side or 400 μL of fresh buffer to the receiver side. Lastly, TPGS was loaded only at the donor side when it was used.

To each transport sample (400 μL), 100 μL of acetonitrile containing 100 μM of testosterone was added as an internal standard and preservative. The resulting mixture was vortexed for 30 s and then centrifuged at 15,000 rpm for 15 min. The supernatant was analyzed by UPLC.

### 3.4. Animals

Male Sprague-Dawley rats (150–170 days old) weighing between 250 and 300 g were from SLEK Lab Animal Center of Shanghai (Shanghai, China). The rats were fasted overnight before the day of the experiment.

### 3.5. Animal study

The procedures were approved by the Animal Ethics Committee of Jiangsu Provincial Academy of Chinese Medicine. The intestinal surgical procedure were modified from previous publication (Liu et al. 2006), in that we perfused four segments of the intestine simultaneously. In this study, the cannulation to the duodenum, jejunum, ileum, and colon, respectively, was connected to a short cannula, which was 1.5 to 2.0 cm long and can be easily disconnected or reconnected to the main perfusion tube. In addition to the careful surgery, caution was also exercised to keep the inlet and outlet cannulate at the same height to avoid gravitational flow. To keep the temperature of the perfusate constant, the inlet cannulate was insulated and kept warm by a 37 °C circulating water bath.

### 3.6. Four-site rat intestinal perfusion experiment

A single-pass perfusion method was used. Four segments of the intestine (duodenum, upper jejunum, terminal ileum, and colon) were perfused simultaneously with a perfusate containing 20 μM icariside II in the presence or absence of 0.5 mg/mL TPGS. To keep the temperature of the perfusate constant, the inlet cannula was insulated and kept warm by a 37 °C circulating water bath. A flow rate of 0.15 ml/min was used, and the perfusate samples were collected every 30 min. The outlet concentrations of icariside II in the perfusate were determined by UPLC (Hu et al. 1998).

### 3.7. Method validation

The UPLC method was used to determine the concentration of icariside II in the transport samples obtained from the four-site perfused rat intestinal model and the Caco-2 model. The conditions for UPLC analysis of icariside II of transport samples were as follows: system, Waters Acquity UPLC with photodiode array detector and Empower software; column, Acquity UPLC BEH C18, 1.7 μm, 2.1 × 50 mm (Waters, Milford, MA, USA); mobile phase A, 100% acetonitrile; mobile phase B, water (v/v); gradient, 0 to 0.9 min, 25% A, 1.0 to 2.1 min, 25% to 60% A, 2.3 to 3.0 min, 25% A; flow rate, 0.4 ml/min; wavelength, icariside II (270 nm); injection volume, 5 μL. The

retention times for icariside II, internal standard were 1.6, 1.4 min, respectively. In general, these methods are selective and reproducible with day to day variability less than 3%. The tested linear response ranges for icariside II were 2.5 to 40 μM, whereas the tested linear response range was 2.5 to 40 μM.

### 3.8. Data analysis

#### 3.8.1. Data analysis in the Caco-2 cell culture model

Rate of transport is obtained from amount transported versus time curve using linear regression. The permeability of a compound is calculated using Eq. (1):

$$P_{app} = \frac{V}{S \times C} \times \frac{dC}{dt} = \frac{1}{S \times C} \times \frac{dM}{dt} \quad (1)$$

where V is the volume of the receiver (typical volume is 2.5 ml), S is the surface area of the cell monolayer (typical surface area is 4.2 $cm^2$), C is the initial concentration, $\frac{dC}{dt}$ is the rate of concentration change in the receiver side, and $\frac{dM}{dt}$ is the rate of drug transport. The rate of drug transport is obtained by linear regression analysis (a Microsoft Excel function).

#### 3.8.2. Data analysis in the rat intestinal perfusion model

In the perfused rat intestinal model, Peff* is a representation of the intestinal membrane permeability, Peff* of the compounds are calculated using the following equation (Eq. 2):

$$Peff* = \frac{1 - C_m/C_0}{4Gz} \quad (2)$$

where Co and Cm are inlet and outlet concentrations, respectively; while Gz, or Graetz number ($G_z = \frac{\pi DL}{2Q}$), is a scaling factor that incorporates flow rate (Q) and intestinal length (L), and diffusion coefficients (D) to make the permeability dimensionless; A is a correction factor for aqueous resistance of the intestine. Cm was adjusted for water flux.

### 3.9. Statistical analysis

All values are expressed as their mean ± S.D. Statistical tests of significance were performed with SPSS Statistics 17.0(SPSS Inc., USA). A value of $P < 0.05$ was considered statistically significant. Data for multiple comparisons was analyzed by One-Way ANOVA and for double comparisons was made by Student's t-tests.

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