Food Emulsifier Polysorbate 80 Increases Intestinal Absorption of Di-(2-Ethylhexyl) Phthalate in Rats

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The aim of the present research was to explore whether food emulsifier polysorbate 80 can enhance the absorption of di-(2-ethylhexyl) phthalate (DEHP) and its possible mechanism. We established the high-performance liquid chromatography (HPLC) method for detecting DEHP and its major metabolite, mono-ethylhexyl phthalate (MEHP) in rat plasma, and then examined the toxicokinetic and bioavailability of DEHP with or without polysorbate 80 in rats. The study of its mechanism to increase the absorption of phthalates demonstrated that polysorbate 80 can induce mitochondrial dysfunction in time- and concentration-dependence manners in Caco-2 cells by reducing mitochondrial membrane potential, diminishing the production of the adenosine triphosphate, and decreasing the activity of electron transport chain. Our results indicated that food emulsifier applied in relatively high concentrations in even the most frequently consumed foods can increase the absorption of DEHP, and its role may be related to the structure and function damages of mitochondria in enterocytes.

Key words: DEHP; food emulsifier; polysorbate 80; toxicokinetic; absorption; P-glycoprotein; mitochondria.

ABBREVIATIONS

DEHP di-(2-ethylhexyl) phthalate
DEP di-ethyl phthalate
EDCs endocrine disrupting chemicals
HPLC high-performance liquid chromatography
MEHP mono-ethylhexyl phthalate
P-gp P-glycoprotein

Phthalates are mostly used as plasticizers in many poly(vinyl chloride) (PVC) consumer products, including biomedical devices, food and beverage packaging, wall coverings, and children’s toys. Di-(2-ethylhexyl) phthalate (DEHP) is one of the most important phthalates in environmental pollutants and the threat factors in human health, because it has been confirmed to cause various toxicities, such as reproductive and developmental toxicities (Lovekamp-Swan and Davis, 2003; Ono et al., 2004), liver hypertrophy (Huber et al., 1996; Lake et al., 1986; Seth, 1982), kidney inflammation (Crocker et al., 1988), and thyroid atrophy in rodents or in human (Meeker et al., 2007). It is universally acknowledged that DEHP belongs to the endocrine disrupting chemicals (EDCs; Nuti et al., 2005).

DEHP and its analogues can migrate from plastic containers to package cooking oil and mineral water (Xu et al., 2010), food (Gonzalez-Castro et al., 2011; Rudel et al., 2011), orange juice (Guo et al., 2010), and baby milk powders (Yano et al., 2005). In the migration study of phthalates from plastic packaging films, diethyl phthalate (DEP), and DEHP in the orange juice were detected their concentrations up to 0.385 and 0.662 μg/ml, respectively. The level of DEHP in orange juice packaged by PVC bottle was about 110-fold higher than the limiting one in drinking water (6 ppb) regulated by United States Environmental Protection Agency (USEPA) (Guo et al., 2010). Dietary exposure to DEHP occurs also through migration from the environment, where it exists as a contaminant in drinking water, fish, and other foods. Although the concentration of DEHP was <0.2 mg/kg in most fish, the concentration was up to 1 mg/kg in fish in the different districts of the United States. In varied food samples, the highest concentrations were found in cheese (35 mg/kg, fat basis) and milk (31.4 mg/l, fat basis) (Anderson et al., 2001; Castle et al., 1988, 1990; Gilbert, 1994; Peijnenburg and Struijs, 2006; Sharman et al., 1994; Staples et al., 2000). Most importantly, DEHP is not permitted as a food additive by any country around the world. However, the emulsifier is a legal food additive in fruit juice, jam, and soft drinks, but some manufacturers in Taiwan used DEHP illegally in the substitution production of the emulsifier to save costs (Yen et al., 2011). So, the human may be exposed to DEHP and other plasti-
cizers by different routes, e.g., ingestion, inhalation, and dermal. Also, it occurs during certain medical procedures, such as blood transfusions and kidney dialysis. Nevertheless, the most probable and significant exposure source to DEHP is food or water, so the main absorption part is in the intestinal tract.

The factors affecting the bioavailability of environmental poisonous substances include the physicochemical properties, such as solubility and permeability. On the other hand, the enterocyte-based metabolism and intestinal efflux transporters (e.g., P-glycoprotein, P-gp) have been proposed to contribute a major barrier to the bioavailability for a number of xenobiotic (Johnson et al., 2001). The oral bioavailability of DEHP in human volunteers is ~25% (30 mg DEHP once or 10 mg/day over 4 days) (Schmid and Schlatter, 1985). DEHP can be converted fleetly into a variety of its metabolites, mono-ethylhexyl phthalate (MEHP), and 2-ethylhexanol, by intestinal pancreatic lipase (Albro et al., 1982; Kluwe, 1982; Rhodes et al., 1986; Sjoberg and Bondesson, 1985; Sjoberg et al., 1985). Kim et al. considered that phthalates were also the P-gp substrates (Kim et al., 2007).

Despite the growing body of literatures on phthalates toxicology, however, very few studies have addressed the effects of food additive surfactants on the toxicokinetic, bioavailability, and metabolism of phthalates in animal models. Polysorbates are hydrophilic nonionic surfactants that they have excellent ability as emulsifiers and lubricants in food products, particularly in ice cream, whipped cream, and nondairy cream alternatives. To make the ice cream smoother and increase its resistance to melting, the concentration of polysorbate 80 in the ice cream is added up to 0.5% (v/v) (Goff, 1997). Furthermore, surfactants such as polysorbate 80 can improve the bioavailability of poorly absorbed drugs by modulating the P-gp in pharmaceuticals research (Cornaire et al., 2000). Several studies have described that polysorbate 80 solution circulated in PVC tubing can increase the amount of DEHP released (Demore et al., 2002; Takehisa et al., 2005).

Based on these factors, our hypothesis is that some food emulsifiers applied in relatively high concentrations in even the most frequently consumed foods may increase the absorption of DEHP by affecting intestinal permeability or other factors. In present work, we proved that polysorbate 80 can increase the absorption of DEHP and focused on studying its possible mechanism by interfering with the efflux microenvironment of enterocytes in rats.

**MATERIALS AND METHODS**

**Materials.** DEHP (CAS 117-81-7), MEHP (CAS 4376-20-9), and di-n-hexyl phthalate (DNHP, CAS 84-75-3) were purchased from TCI (Tokyo, Japan). Polysorbate 80 (CAS 9005-65-6), 5.5′,6,6′-tetrachloro-1,1′,3,3′-tetrachlorobenzimidazolylcarbocyanine iodide (JC-1, CAS 47729-63-5), and dimethyl sulfoxide (DMSO, CAS 67-68-5) were acquired from Sigma-Aldrich (St Louis, MO). The chemical structures of DEHP, MEHP, DNHP, and polysorbate 80 are shown in Figure 1.

**Animals.** Sprague Dawley rats (weighing 220–260 g) were purchased from Southeast University Experimental Animal Center and maintained in an environmentally controlled room (23 °C ± 3 °C, 12 h dark-light cycle) with free access to standard animal laboratory food for 7 days before experiments at Animal Laboratory of Nanjing Normal University. Water was supplied ad libitum from glass bottles. Animal experiments were assuming the “Principles of laboratory animal care” (NIH publication 86-23, revised 1986) and local regulations. Furthermore, all experiments were approved and supervised by the Animal Care and Use Committee and the Animal Ethics Committee at Nanjing Normal University.

**High-performance liquid chromatography analyses.** A high-performance liquid chromatography (HPLC) method was developed for the detection of DEHP and its primary metabolite MEHP in rat blood. HPLC analysis was carried out using an Agilent 1100 LC system (Santa Clara, CA) composed of the following: a Model 515 pump, a Model 996 photodiode array detector equipped with a Millennium 32 software, and a Model 7125 sample injector equipped with a 20-μl loop. HPLC separation was carried out with UV detection at a wavelength of 235 nm on an Agilent C18 column (75 mm × 4.6 mm, 3.5 μm) and a precolumn at a flow rate of 1 ml/min using a mobile phase of acetonitrile:0.1% H3PO4 = 90:10 (vol/vol). The mobile phase was filtered through a 0.22 μm nylon membrane filter and ultrasonically degassing before it was used. Column temperature was at room temperature, and the injection volume was 10 μl. DNHP, as the internal standard (IS), is utilized to normalize peak areas from each injection in a sample set (Cobellis et al., 2003).

Stock solutions containing DEHP or MEHP were prepared by dissolving a weighed amount of substance in acetonitrile. Standard solutions were prepared by dilution of the above stock solutions with the mobile phase and by varying the concentration in the range 0.05 ± 5 mg/ml.

**Toxicokinetic and bioavailability studies.** For the toxicokinetic study, 10 rats were fasted overnight before administration and divided randomly into two groups (n = 5). One group rats received DEHP suspension with 0.5% in CMC-Na at a dose of 500 mg/kg by intragastrical (ig) administration. Another group rats received DEHP at a dose of 500 mg/kg with 0.25% polysorbate 80 (25 mg/kg) by ig. Whole blood samples (~0.3 ml) were withdrawn from the orbital sinus into the heparinization centrifuge tubes at 15, 30, 60, 90, 120, 180, 240, 300, 360, 720, and 1440 min following ig. The rats were given normal saline to make up the blood loss during experimentation. The plasma sample was separated by centrifugation at 1500 × g for 10 min.
within 30 min after the collection of blood samples and stored at −70°C before HPLC analysis.

Toxicokinetic parameters were determined by the 3p97 software provided by the Chinese Pharmacological Society. The data were represented by the following primary parameters: area under concentration-time curve (AUC), plasma half-life ($t_{1/2}$), the peak concentration ($C_{\text{max}}$), and time of peak concentration ($T_{\text{max}}$). $C_{\text{max}}$ and $T_{\text{max}}$ were determined directly from experimental observations. Areas under concentration-time curves from time zero to infinity (AUC$_{0-\infty}$) were estimated using the log-linear trapezoidal method.

Relative bioavailability ($F_{\text{rel}}$) is used to compare the bioavailability of DEHP and MEHP with or without polysorbate 80 following ig in the systemic circulation. $F_{\text{rel}}$ was calculated by the following formula:

$$F_{\text{rel}}(\%) = 100 \times \frac{\text{AUC}_A \times \text{Dose}_B}{\text{AUC}_B \times \text{Dose}_A}$$

AUC$_A$ and AUC$_B$ represent respectively the area under the blood concentration-time curve of DEHP and MEHP with or without polysorbate 80; Dose A and Dose B mean the dose of DEHP with or without polysorbate 80 following ig administration, respectively.

Caco-2 cell culture. The human colon carcinoma (Caco-2) cells (passage 30–35, American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 5mM glutamine, supplemented with 12% fetal bovine serum (FBS), and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO$_2$. Caco-2 cells were seeded onto six-well plates (Corning, New York) at a density of $5 \times 10^4$ cells/ml.

Caco-2 cell viability. To formulate the least cytotoxic, polysorbate 80 was screened for toxicity on Caco-2 cells. The cells were harvested with trypsin for 5 min at 37°C, and resuspended in complete medium. The cells were seeded in 96-well plates (Corning) at a density of $5 \times 10^4$ cells/ml and allowed to attach until a confluence of 70% was reached. Then, the medium was changed to 100 ml of FBS-free medium containing polysorbate 80 at concentrations ranging from 0.05 to 0.5%. The blank culture medium was invoked as a control. After the treated cells were incubated for 3 h at 37°C in an incubator (Series 8000 WJ, Thermo Scientific, Waltham, MA), 10 μl of the WST-1 solution was added to each well and further incubated for 4 h. Afterward, the optical absorbance was measured using a microplate reader (Model 680, Bio-Rad, Hercules, CA) at 450 nm to determine cell viability. The percentage viability of the cells was calculated as the ratio of absorbance of triplicate readings with respect to the absorbance of control wells using the formula:

$$\text{cell viability (\%)} = \frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{control}}}. \quad (2)$$

All experiments were carried out in triplicate, and the mean viability of the three ± standard deviation was determined.

Mitochondrial membrane potential (MMP) studies. The effect of polysorbate 80 disturbing mitochondrial membrane potential (MMP, $\Delta \Psi_m$) was evaluated using JC-1 MMP Assay Kit. Caco-2 cells were seeded at $5 \times 10^5$ cells/well in six-well dishes (Corning) in a CO$_2$ incubator overnight at 37°C. The cells were treated with serum-free medium (a negative control), sodium azide (NaN$_3$, a positive control, 10μM), 0.05%, and 0.125% polysorbate 80 for 2 or 3 h, respectively. After the supernatant was removed, the cells were incubated with 10 μl JC-1 reagents for 30 min at 37°C in darkness. The Caco-2 cells were gently rinsed with PBS three times prior to photographing immediately under fluorescence microscopy (AX10, Zeiss, Germany). The detection wavelengths of JC-1 aggregates are 540 nm (excitation) and 570 nm (emission), and the detection wavelengths of JC-1 monomers are 485 nm (excitation) and 535 nm (emission), respectively. The ratio of red/green (R/G) fluorescence for each sample was calculated by ImageJ software (version 1.45) (Wang et al., 2012). The relation between MMP
(ΔΨ$_m$) and the ratio of R/G was expressed using the formula:

$$
\Delta \Psi_m = \frac{\text{the ratio of R/G}_{\text{treatment}}}{\text{the ratio of R/G}_{\text{control}}}.
$$

Mitochondrial respiration rate studies. The oxygen consumption rate (OCR, pmol/min), an indicator of mitochondrial respiration, was measured using a Seahorse Bioscience XF96 instrument (Seahorse Bioscience, North Billerica, MA). The optimal cell density and the working concentrations of the used compounds have been determined by a preliminary test before the formal experiment. The Caco-2 cells were collected and seeded at a density of 4.40 × 10$^5$ cells/well in 96-well plates coated with polylysine. The cells were cultured to attachment with 5% CO$_2$ at 37°C for 24 h, and then treated with different concentration of polysorbate 80 (0.025, 0.05, 0.125, and 0.25%) for 1, 2, and 3 h, respectively. The basal OCR was started to assay after changing the growth medium into an assay medium in each well and preincubated at 37°C without CO$_2$ for 1 h. A cartridge was configured as individual well “plungers” which comprised the optical fluorescent O$_2$ sensors for 96-well plates. After softly descending into the wells, the plungers formed a chamber comprising an extremely small volume (<3 µl) of medium above the cell monolayer. After the basal OCR was determined, 1µM oligomycin and 0.3µM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) were added into the cell wells. The experiment was performed for 1.5 h to detect the oxidative capacity of the cells under different conditions. The number of cells present in a well is not relevant, because the population of cells is the same at each time assay, i.e., paired comparison design.

Data analysis and statistics. Results are expressed as means ± standard deviation (SD). Data were analyzed by one-way analysis of variance, and differences among the means of groups were analyzed by an unpaired, two sided Student’s t-test. Differences were considered significant at p < 0.05 and very significant at p < 0.01, respectively.

RESULTS

HPLC Analysis

The peaks of MEHP, DEHP, and polysorbate 80 were lacking in interferences arising from other plasma components. MEHP, DEHP, polysorbate 80, and DNHP (IS) were marked by retention times of 3.4, 12.4, 20, and 24 min, respectively. A good linear relationship was obtained for MEHP and DEHP with concentration ranging from 5 to 1000 ng/ml (y = 4.40 × 10$^{-3}$x + 0.025, R$^2$ = 0.9998, x—concentration of DEHP in plasma, y—peak areas of DEHP), (y = 4.40 × 10$^{-3}$x + 0.025, R$^2$ = 0.9998, x—concentration of MEHP in plasma, y—peak areas of MEHP), respectively. The lowest detectable limit (LDL) for MEHP and DEHP was 100 and 100 ng/ml, respectively. The coefficient of variation (CV) of both MEHP and DEHP for inter- and intraday precision of low, medium, and high concentrations (5.0, 50.0, and 500.0 ng/ml) was <10.0%. The results of intra- and interday analysis indicated that the method was accurate, reliable, and reproducible.

There was no interference from the sample matrix indicating the specificity of the method. Satisfactory recoveries (92–102%) were observed over a linear range of 25–800 ng/ml. Method precision was 0.6% RSD at a polysorbate 80 concentration of 200 ng/ml and the mean recovery was 98.0 ± 0.7%. The method demonstrated acceptable sample stability at ambient temperature up to 6 days (<10% difference; data not shown).

Toxicokinetic and Bioavailability Studies of DEHP with or without Polysorbate 80

The main toxicokinetic parameters and the plasma concentration-time profile of DEHP and its metabolite MEHP in rats are shown in Table 1 and Figure 2. After oral administration DEHP (500 mg/kg) with or without food emulsifier polysorbate 80 (25 mg/kg), the maximum concentration (C$_{\text{max}}$, µg/ml) of DEHP was 26.35 ± 8.31 and 17.28 ± 2.18; AUC$_{0-1440}$ (min µg/ml) were 4 903 ± 1 882 and 2582 ± 603.6, respectively.

We also detected the plasma concentration-time profile of MEHP as the most important metabolites of DEHP and analyzed its toxicokinetic parameters. After oral administration DEHP (500 mg/kg) with or without 0.5% polysorbate 80, the maximum concentration (C$_{\text{max}}$, min µg/ml) of MEHP was 55.19 ± 2.77 and 32.51 ± 9.31, AUC$_{0-1440}$ was 11 264 ± 541.7 and 5 180 ± 750.6, respectively.

Caco-2 Cell Viability

The effect of polysorbate 80 on the Caco-2 cell viability used the WST-1 assay is illustrated in Figure 3. Polysorbate 80 at lower concentrations (0.05–0.125%) did not show significantly cytotoxicity. Then, the viability of Caco-2 cells decreased by 0.25% polysorbate 80 incubation. However, polysorbate 80 at 0.5% concentration would result in the decrease of the viability to 25% in Caco-2 cells. Therefore, the concentration of polysorbate 80 applied in the following experiment did not exceed 0.25% to make sure that the cell survival was not notably inhibited compared with group control.

MMP Studies

MMP is a key parameter of mitochondrial structure and function and can be detected with a fluorescence probe, JC-1. The decreased of MMP is the characteristic event of mitochondrial disorders. MMP is assessed by the variance ratio of JC-1 aggregate (red) two monomers (green) fluorescence intensity. The lower R/G ratio indicates a decrease of MMP. In the positive control groups (Na$_3$NO, 10µM), with the time increased from 1 to 3 h, the intensity ratio of JC-1 R/G fluorescence decreased from 4.7% (p < 0.05) to 12.28% (p < 0.01) compared with the same time point in the negative control group. While incubating...
POLYSORBATE 80 INCREASES ABSORPTION OF DEHP

FIG. 2. Mean plasma concentration-time profiles of DEHP (A) and MEHP (B) following DEHP (500 mg/kg, ig) with or without 0.5% Polysorbate 80 (25 mg/kg, ig) in rats (n=5). (●) DEHP with Polysorbate 80 and (■) DEHP without Polysorbate 80.

TABLE 1

The Toxicokinetics Parameters of DEHP Administrated Following Intragastrical Administration (ig) in Rats

<table>
<thead>
<tr>
<th></th>
<th>DEHP without Polysorbate 80</th>
<th>DEHP with Polysorbate 80</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEHP mg/kg</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Polysorbate 80 mg/kg</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Polysorbate 80 %</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>t</strong>&lt;sub&gt;1/2&lt;/sub&gt; min</td>
<td>63.10 ± 16.14</td>
<td>68.61 ± 9.37</td>
</tr>
<tr>
<td><strong>C</strong>&lt;sub&gt;max&lt;/sub&gt; μg/ml</td>
<td>17.28 ± 2.18</td>
<td>32.51 ± 9.31</td>
</tr>
<tr>
<td><strong>T</strong>&lt;sub&gt;max&lt;/sub&gt; min</td>
<td>72.00 ± 16.43</td>
<td>72.00 ± 16.43</td>
</tr>
<tr>
<td><strong>AUC</strong>&lt;sub&gt;tmin&lt;/sub&gt; min μg/ml</td>
<td>2582 ± 603.6</td>
<td>5180 ± 750.6</td>
</tr>
<tr>
<td><strong>F</strong>&lt;sub&gt;rel&lt;/sub&gt; %</td>
<td>190</td>
<td>217</td>
</tr>
</tbody>
</table>

Note: **t**<sub>1/2</sub>, plasma half-life; **C**<sub>max</sub>, maximum concentration; **T**<sub>max</sub>, time to reach **C**<sub>max</sub>; **AUC**<sub>tmin</sub>, area under the blood concentration versus time curve; **F**<sub>rel</sub>, relative bioavailability. mean ± SD, n=5. *p < 0.05, **p < 0.01, versus DEHP, Student’s *t*-test.

FIG. 3. The cell viability of Caco-2 cells after exposure to Polysorbate 80 concentrations of 0.05% to 0.5%.

with 0.05% polysorbate 80 for 1 h, the ratio of R/G fluorescence of the Caco-2 cells was statistically reduced by 2.3%. At longer times at 3 h of incubation, a lower decrease of 3.5% compared with the negative control group was found. Polysorbate 80 concentrations at 0.125 and 0.05% induced the reduction in the ratio of R/G fluorescence in Caco-2 cell to 10.28% (p < 0.01) and 3.6% (p < 0.05), respectively. So, polysorbate 80 caused the decrease of MMP in a concentration-dependent manner.

These results confirmed that polysorbate 80 could reduce MMP and induce mitochondrial dysfunction in time- and concentration-dependence manners.

Mitochondrial Respiration Rate Studies

We measured OCR to prove further that polysorbate 80 induced mitochondrial damage of Caco-2 cells. Oligomycin can inhibit state-3 respiration completely and used to detect the part of oxygen consumption applies for adenosine triphosphate (ATP) synthesis in analysis of mitochondrial respiration rate. As Figure 6B shown, after the cells were treated only for 1 h with 0.05% polysorbate 80, OCR values of basal respiration had been significantly reduced by 16% (p < 0.05). When the treatment time was prolonged to 2 h, basic OCR reduced as lower as by 27% (p < 0.01). After exposing the same concentration of polysorbate 80 for 3 h, basic OCR was obviously declined 38% (p < 0.01). This data indicated that polysorbate 80 retained the basic respiration of mitochondria in a time-dependent manner.

After 1 h treatment with 0.05% polysorbate 80 in Caco-2 cells, the AUC of OCR was decreased by 42% compared with the negative control. When the treatment time was extended to 2 and 3 h, the reductions were 59 and 68% separately (p < 0.01; Fig. 6C). The results indicated that the production of ATP in the
FIG. 4. The time-dependence effect of polysorbate 80 (0.05%) on the MMP of Caco-2 cells. (A) – (F) Representative images of Caco-2 cells under fluorescence microscope. (A) a negative control for 1 h, (B) NaN3 (10 μM, as a positive control) for 1 h, (C) Polysorbate 80 for 1 h, and (D) a negative control for 3 h, (E) NaN3 (10 μM, as a positive control) for 3 h, (F) Polysorbate 80 for 3 h. (G) The quantitative analysis of fluorescence microscopy images JC-1 red/green fluorescence intensity ratio by Image J (mean ± SD, n=8). (*) p < .05 and (**) p < .01, compared with the negative control group at the same time point, respectively.

mitochondria of Caco-2 cells was diminished by polysorbate 80 in a time-dependent manner.

As shown in Figure 7, although the Caco-2 cells were treated for 1 h with different concentrations of polysorbate 80, the basic OCR was reduced from 16% (p < 0.01) to 55% (p < 0.01) as the concentration increased from 0.025 to 0.25%. Taking the viability of the Caco-2 cells into consideration, the result of 0.25% polysorbate treatment was insignificant. Thereinto, when the concentration of polysorbate 80 was 0.05%, the reduction of the basic OCR was 20% (p < 0.01). Then, after the concentration was raised to 0.125%, basic OCR was reduced by 42% (p < 0.01), whereas OCR of the positive control was lower only declined 25% (p < 0.01). We can illustrate that polysorbate 80 influences the basic respiration not only in a time-dependent manner, but also in a dose-dependent manner.

Furthermore, NaN3 as a positive control inhibited the ATP synthesis in mitochondrial state-3 respiration, and expressed an understandable decline by 41% (p < 0.01, with negative control) on the AUC of OCR. Polysorbate 80 (concentrations from 0.025 to 0.05%, and 0.125%) induced expected reduction in the AUC of OCR from 22 to 42%, and 61%, respectively (Fig. 7). The above analysis further revealed that polysorbate 80 could injure the mitochondrial function in Caco-2 cells.

FIG. 5. The concentration-dependence effect of polysorbate 80 (0.05%, 0.125%) on the MMP of Caco-2 cells. (A)–(D) Representative images of Caco-2 cells under fluorescence microscope incubated for 3 h with (A) a negative control, (B) NaN3 (10 μM, as a positive control), (C) Polysorbate 80 (0.05%), and (D) Polysorbate 80 (0.125%). (E) The quantitative analysis of fluorescence microscopy images JC-1 red/green fluorescence intensity ratio by Image J (mean ± SD, n=8). (*) p < .05 and (**) p < .01, compared with the control group.
FIG. 6. The effect of polysorbate 80 on mitochondrial respiration in Caco-2 cells at different times used the Seahorse extracellular flux analytical essay. (A) After incubating separately with polysorbate 80 for 1 h, 2 h and 3 h, oxygen consumption rate (OCR) exposed sequentially to each modulator of mitochondrial activity (Oligomycin, FCCP and Rotenone) for another 1.5 h. (B) Basal cellular respiration treatment with polysorbate 80 for the different time group. (C) Effect of polysorbate 80 on Oligomycin-sensitive OCR. The OCR difference was calculated as the measured OCR level minus the baseline OCR level (at the 17th minute), which was expressed as area under the curve (AUC) of point 3 to point 6 in A after the first injection of Oligomycin. (D) Effect of polysorbate 80 on FCCP-stimulated OCR is shown. The OCR difference was calculated as the measured OCR level minus the baseline OCR level (at the 17th minute), which was expressed as AUC of point 6 to point 9 in A after the injection of FCCP. Data in (B - D) represent mean ± SD of three independent experiments. (*) \( P < .05 \) and (**) \( P < .01 \) versus the negative control.

DISCUSSION

The Validation of HPLC Analyses

To research the effect of food emulsifier polysorbate 80 on the toxicokinetic of phthalates, we developed a simple and rapid HPLC method for analyzing the concentration of food emulsifier polysorbate 80 and EDCs of phthalates including DEHP and its major metabolite MEHP in rat plasma. There were some reports on the determination of polysorbate 80 (Adamo et al., 2010; Hu et al., 2003), or DEHP and MEHP in biological specimens by HPLC (Cobellis et al., 2003; Mitani et al., 2003; Sircar et al., 2008). Our method is validated with respect to the linearity range, the limit of detection, specificity, inter- and intraday precision. The retention times of MEHP, polysorbate 80, DEHP, and DNHP (IS) are 3.4, 12.4, 20, and 24 min, respectively, and with good resolution and without any interference from endogenous plasma constituents at or near the retention time of those targeting materials. Therefore, the satisfactory specificity of the proposed method is obtained. The validated method has been successfully applied to the toxicokinetic of DEHP in rat plasma samples in our study.

Pearson et al. found that polysorbate 80 was actively involved in extracting DEHP from plasticized PVC containers, especially in combination with ethanol (Pearson and Trissel, 1993). Demore et al. reported that polysorbate 80 can lead the release of DEHP from PVC bags into an intravenous solution of drug (Demore et al., 2002). In order to avoid the migrating of DEHP in the blood sample from PVC, we used a glass tube instead of PVC tube in contact with organic solvents in our study.

Food Emulsifier Polysorbate 80 may Increase the Bioavailability of DEHP by Improving the Absorption of DEHP and MEHP

There are some studies on the toxicokinetic and bioavailability of DEHP over the last about 40 years (Chang-Liao et al., 2013; Daniel and Bratt, 1974; Rhodes et al., 1986). In these literatures, the dosage of DEHP with a single exposure by oral administration in the rat was in the range of 50–2000 mg/kg. On the other hand, Koo et al. considered that the toxicokinetic of DEHP should be based on DEHP and MEHP in serum in rats treated with DEHP (40, 200, or 1000 mg/kg, ig) for risk assessment applications (Koo and Lee, 2007). Moreover, MEHP is regarded as responsible for much of DEHP’s toxicity (Choi et al., 2012; Greiner et al., 2012). So, we designed the dose of 500 mg/kg for the toxicokinetic study and confirmed that the concentrations of DEHP and MEHP represented the blood poison concentration.
FIG. 7. The effect of polysorbate 80 on mitochondrial respiration in Caco-2 cells at different concentrations used the Seahorse extracellular flux analytical essay. (A) Oxygen consumption rate (OCR) of Caco-2 cells exposed sequentially to each modulator of mitochondrial activity (Oligomycin, FCCP and Rotenone) in the presence of polysorbate 80 at different concentrations for 1.5 h. (B) Basal cellular respiration for each concentration used. (C) The effect of polysorbate 80 on Oligomycin-sensitive OCR. The OCR difference was calculated as the measured OCR level minus the baseline OCR level (at the 17th minute), which expressed as area under the curve (AUC) of point 3 to point 6 in A after the first injection of Oligomycin. (D) Effect of polysorbate 80 on FCCP-stimulated OCR. The OCR difference was calculated as the measured OCR level minus the baseline OCR level (at the 17th minute), which expressed as AUC of point 6 to point 9 in A after the injection of FCCP. Data in (B - D) represent mean ± SD of three independent experiments. (*) \( p < .05 \) and (**) \( p < .01 \) versus the negative control.

Recently, Chang-Liao et al. found the oral bioavailability of DEHP was ∼7% used UPLC-MS/MS method in rats (Chang-Liao et al., 2013). Pollack et al. had investigated that the systemic availability of DEHP was 13.6% following single oral administration (Pollack et al., 1985). The low bioavailability of DEHP may have a variety of reasons, such as, having not measured its main metabolite MEHP. Because of the presence of the esterase responsible for its metabolism in the intestine, DEHP may be absorbed in its primary metabolites MEHP and 2-ethylhexanol (Daniel and Bratt, 1974).

Polysorbate 80 as a food emulsifier additive is employed in some food products in a higher concentration, such as, ice cream, nondairy cream, and whipped cream. The quantity of polysorbate 80 as high as 0.5% (vol/vol) has been used in ice cream (Goff, 1997). We considered that the dosage of polysorbate 80 in some food products could influence the absorption of phthalates from the gastrointestinal tract. The present results showed that DEHP with polysorbate 80 had higher plasma concentrations of DEHP and its metabolite MEHP as compared with DEHP with 0.5% CMC-Na in rats. The relative bioavailability of DEHP and MEHP was a significant increase to 1.9 and 2.2 times, respectively.

Polysorbate 80 is also known as an inhibitor of efflux transporter P-gp for some P-gp substrates (Rege et al., 2001). Its inhibiting effect may be part of mechanisms to increase the bioavailability of DEHP by improving the absorption of DEHP and MEHP. Differing from P-gp specificity inhibitor, these emulsifiers can moderate P-gp efflux pump by the ways of indirectly and nonspecifically. Polysorbate 80 may embed in the cell membrane, breach the composition of membrane glycopro-
teins, inhibit P-gp efflux pump, and enhance the intestinal absorption. Therefore, polysorbate 80 can increase the bioavailability of some P-gp substrates. Recently, Kim et al. reported that phthalates, including DEP, DBP, and DEHP, can be considered as the P-gp substrates (Kim et al., 2007). P-gp is an ABC-transporter of the MDR/TAP subfamily, extensively distributed in the intestinal epithelium and blood-testis barrier. The toxicokinetic of environmental toxicants has extensively been influenced by P-gp-mediated efflux in absorption, distribution, and clearance (Abu-Qare et al., 2003; van Tellingen, 2001). The intestinal P-gp efflux pump has been proposed to contribute a major barrier to the oral bioavailability for a number of environmental toxicants.

**Structure and Function of Mitochondria**

There were a few studies on the effect and mechanism of polysorbate 80 on P-gp efflux pump focused on the structure of cell membrane (Cornaire et al., 2004; Zhang et al., 2003). Recently, we proposed the conception of the intracellular efflux microenvironment, including lipid raft in the cell membrane and some organelles, such as mitochondria. The intracellular efflux microenvironment plays an important role in the efflux of smaller molecule chemicals and environmental toxicants (Wang et al., 2012). We hypothesized that the ATP diminished was caused by polysorbate 80 with the damage to the mitochondrial structure. Then, the accumulation of DEHP intracellular was enhanced by P-gp efflux pump in effectiveness without energy support. To clarify our hypothesis, the relationship between the mitochondrial dysfunction and energy supply should be explored, so we also determined the MMP (Figs. 4 and 5) and the OCRs change in state-3 respiration (phosphorylating) with polysorbate 80 treatments, which expressed as the AUC of point 3 to point 6 in Figures 6 and 7.

MMP is one of the most important parameters of mitochondria functional status. Hence, we examined the effect of polysorbate 80 on the depolarization of the mitochondrial membrane in Caco-2 cells with fluorescent probe JC-1. The dye probe with the ability selectively to enter mitochondria was used as an indicator of $\Delta \Psi_m$. J-aggregates, known as JC-1 spontaneous form complex, exhibits an intense red fluorescence with normal MMP in the healthy cells. Meanwhile, the monomeric form of JC-1 shows a green fluorescence with low $\Delta \Psi_m$ in the unhealthy cells. Statistically significant variation in $\Delta \Psi_m$ was observed after 3 h of treatment time with 0.125% polysorbate 80. The level of green fluorescence was enhanced in a time- and concentration-dependent manner (Figs. 4 and 5) with the treatment of polysorbate 80 in the Caco-2 cells, which demonstrated the dissipation of $\Delta \Psi_m$ and caused mitochondria dysfunction.

**Mitochondrial Respiration Rate**

As mentioned above, our results from analysis of mitochondrial respiration rate suggested that polysorbate 80 had eliminated the energy in mitochondrial state-3 respiration not only in a time-dependent manner but also in a dose-dependent manner. It further illustrated that polysorbate 80 can injure the mitochondrial function in Caco-2 cells.

The production of ATP in mitochondria depends chiefly on the oxidation of substrates within the tricarboxylic acid cycle. Afterward, electron carriers, that are NADH and FADH2, are oxidized by electron transport complexes I and II, respectively (DeBerardinis et al., 2008). Then the respiratory complexes III and IV are transferred the liberated electrons to the terminal electron acceptor by prosthetic groups (Adam-Vizi, 2005). Meanwhile, complexes I, III, and IV transport the protons from the matrix to mitochondrial intermembrane space (Murphy, 2009). A proton-motive force is originated that consist of an electrical gradient ($\Delta \Psi_m$) and a proton gradient transferring the mitochondrial intermembrane. Owing to $\Delta \Psi_m$ is accounted for 80–85% of a proton-motive force and could be easily measured, $\Delta \Psi_m$ is often inaccuracy used in stand of a proton-motive force.
et al. (2012). Although the protons move through the mitochondrial intermembrane back to the matrix, the ATP synthase drives the formation of ATP. In summary, ATP production is coupled to the transfer of electrons, called the coupled respiration (Harper et al., 2008).

Our results indicated that mitochondrial respiratory function was evidently disturbed by polysorbate 80 (Figs. 6 and 7) in Caco-2 cells. Because the external environment oxygen level stayed consistently stable, the change of OCR was mainly affected by polysorbate 80, which reflected the effect in mitochondrial respiration responsible for the production of ATP through the reduction of oxygen (Figs. 6C and 7C).

Oligomycin is an inhibitor of ATP synthesis used to prevent state-3 (phosphorylating) respiration. It can generate the decrease of OCR by means of blocking the proton channel of the ATP synthase (complex V). The decrease of OCR compared with basal supplies the coupling efficiency. Polysorbate 80 decreased the coupling efficiency of oligomycin, which is accordance with an increase in proton leak and a decrease in ATP linked respiration (Figs. 6C and 7C).

FCCP is a mitochondrial oxidative phosphorylation inhibitor and an uncoupling agent, which disrupted ATP synthesis by transporting hydrogen ions across the mitochondrial membrane instead of the proton channel of ATP synthase (complex V), increasing the OCR. Besides, this maximal respiration is controlled by electron transport chain activity; furthermore, a decreased in FCCP uncoupled respiration could be the result of reduced MMP, consistent with JC-1 MMP assay. Based on these findings, we extrapolated that polysorbate 80 induced the injured in the electron transport chain and possibly caused an intracellular energy supply deficiency in Caco-2 cells.

CONCLUSION

To our knowledge, this is the first study to evaluate the toxicokinetic and bioavailability of DEHP with or without polysorbate 80 in rats. In the present study, polysorbate 80 increasing the bioavailability of DEHP may be resulted from enhancing the absorption by improving the permeability and inhibiting the intracellular microenvironment of P-gp-mediated efflux, and the role of food emulsifier maybe relates to the structure and function damages of mitochondria (Fig. 8). Our results support the novel hypothesis that food emulsifier applied in relatively high concentrations in even the most frequently consumed foods can increase the absorption of phthalates, especially DEHP.

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REFERENCES


