Increased systemic exposure to *Rhizoma coptidis* alkaloids in lipopolysaccharide-pretreated rats due to enhanced intestinal absorption

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Running title: LPS increased the absorption of *Rhizoma coptidis* alkaloids

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Abbreviations: CYPs, cytochrome P450 enzymes; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LPS, lipopolysaccharide; MS/MS, tandem mass spectrometry; NADPH, deaminotriphosphopyridine nucleotide; P-gp, p-glycoprotein; TCM, traditional Chinese medicine; UDPGA, uridine-5'-diphosphoglucuronic acid; UGTs, UDP-glucuronosyltransferase isoforms.
Abstract

*Rhizoma coptidis* is a rhizome commonly used in traditional Chinese medicine. Interestingly, after the oral administration of *Rhizoma coptidis* extract, the plasma concentrations of its effective alkaloid constituents are so low that their systemic therapeutic actions cannot be explained. This study aims to investigate the influence of lipopolysaccharide (LPS) on the pharmacokinetics of the *Rhizoma coptidis* alkaloids. Pharmacokinetic experiments were performed on rats; both *in vitro* absorption and efflux experiments were carried out with everted rat gut sacs, while *in vitro* metabolism experiments were conducted with rat liver microsome and intestinal S9. Mucosa changes were evaluated by light microscopy and transmission electron microscopy. The results showed that in rat plasma, LPS pretreatment increased the systemic exposure of the alkaloids. LPS pretreatment increased the *in vitro* absorption of the alkaloids and decreased their efflux. The efflux of vinblastine as well as rhodamine123, p-glycoprotein (P-gp) substrates, was also decreased. The absorption of fluorescein isothiocyanate-labeled dextran (FD-4; average molecular mass 4 kDa), a gut paracellular permeability probe, was not influenced. Obvious damages were observed in mucosa, but the tight junction between epithelial cells remained intact. Intestinal, instead of hepatic, alkaloid metabolism was diminished. These findings indicate that LPS pretreatment increases the systemic exposure of the alkaloids through the enhancement of their absorption, which was related to the decreased intestinal efflux and metabolism. The results add to the understanding of why *Rhizoma coptidis* is active despite the low plasma
concentrations of the *Rhizoma coptidis* alkaloids measured in normal subjects and experiment animals.
Introduction

*Rhizoma coptidis* (Huang Lian) is the dried rhizome of several medicinal plants of the family Ranunculaceae, such as *Coptis chinensis* Franch. It is commonly used in traditional Chinese medicine (TCM) given its various pharmacological effects including anti-bacterial, anti-fungal, anti-viral, anti-diabetes, anti-cardiovascular diseases, anti-cancer, and anti-inflammatory activities (Yu et al., 2006). It yields alkaloids such as berberine, coptisine, palmatine, and jatrorrhizine (Chen et al., 2008) (Fig. 1). These alkaloids are the effective constituents in *Rhizoma coptidis*, but berberine is the primary compound.

The plasma concentrations of the alkaloids are very low after oral administration of *Rhizoma coptidis* extract (Yu et al., 2007). Likewise, berberine exhibits extremely low plasma concentration after oral administration (Zuo et al., 2006; Liu et al., 2009). Researchers have proposed mechanisms for the low plasma concentrations of these alkaloids: (1) the alkaloids are metabolized in the intestine (Liu et al., 2010); (2) absorbed alkaloids are pumped out by intestinal (Zhang et al., 2011) or hepatic (Tsai and Tsai, 2004) p-glycoprotein (P-gp); (3) absorbed alkaloids are quickly and extensively metabolized after absorption (Tsai and Tsai, 2004; Zuo et al., 2006; Yang et al., 2010); and (4) absorbed alkaloids are widely distributed in tissues (Ma et al., 2010; Liu et al., 2010). The low plasma concentrations of the alkaloids are insufficient indicators of their *in vivo* bioactivities. The alkaloids may present their systemic therapeutic actions by modulating effectors in the gut (Lu et al., 2009), via generated metabolites because they are both abundant (Zuo et al., 2006) and active (Li et al., 2011), or
by the relatively dominant tissue distribution of the alkaloids themselves (Ma et al., 2010; Liu et al., 2010). However, all the aforementioned pharmacokinetic studies were performed on normal subjects and experimental animals, and less is known about systemic exposure to *Rhizoma coptidis* alkaloids in pathological cases, except for diabetic rats (Yu et al., 2010).

Oral *Rhizoma coptidis* extract and its alkaloid constituents are broadly applied in clinics for the treatment of infectious, gastrointestinal, cardiovascular diseases, and diabetes mellitus (Xu et al., 2004; Vuddanda et al., 2010). Both the extract and its alkaloid constituents also show promise as novel anti-neoplastic agents (Tang et al., 2009). On one hand, inflammation is observed in all the above mentioned diseases (Pandolfi et al., 2009; Goldfine et al., 2011; Fearon and Fearon, 2008; Grivennikov and Karin, 2011). On the other hand, both drug-metabolizing enzymes and transporters, which determine the fate of drugs *in vivo*, are regulated in these diseases (Morgan et al., 2008). Given that the *Rhizoma coptidis* alkaloids are substrates of P-gp (Zhang et al., 2011) and are mainly eliminated by metabolism (Yang et al., 2010), we assume that the pharmacokinetics of the alkaloids changes under inflammation compared with that in normal conditions. We hypothesize that this change may be critical to elucidating why *Rhizoma coptidis* cures the above mentioned inflammation-related diseases despite the low plasma concentrations of the alkaloids measured in normal subjects and experimental animals.

Lipopolysaccharide (LPS), the outer membrane molecule of gram-negative bacteria, has often been used to establish both *in vitro* and *in vivo* inflammatory experimental models (Morgan et al., 2008). It reportedly influences the pharmacokinetics of numerous drugs (Yang and Lee, 2008). In the present study, therefore, we aim to assess the effect of LPS
pretreatment on the pharmacokinetics of the *Rhizoma coptidis* alkaloids, and study the underlying mechanisms.
Materials and Methods

**Materials.** *Rhizoma coptidis* (*Coptis chinensis* Franch), which was produced according to *The preparing standardization of the crude Traditional Chinese Medicine of Shanghai (2008 edition)*, was purchased from Shanghai Kang Qiao Herbal Pieces Co., Ltd (China), which is a GMP-certificated manufacturer. The authentication of the herb was performed by Prof. Zhi-Li Zhao of the Department of Botany, Shanghai University of Traditional Chinese Medicine via comparison with appropriate voucher specimens and analysis of both the physical and chemical properties according to *The Pharmacopoeia of People’s Republic of China (2010 edition)*. All *Rhizoma coptidis* alkaloid standards (except coptisine) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The coptisine standard was purchased from Wako Pure Chemical Ind., Ltd. (Japan). Vinblastine and vincristine were the products of USP (Rockville, USA). A bicinchoninic acid (BCA) protein assay kit was obtained from the Shanghai Usen Biological Technology Co. (Shanghai, China). LPS, rhodamine123 (Rh-123), fluorescein isothiocyanate-labeled dextran (FD-4; average molecular mass 4 kDa), phenacetin standard, deaminotriphosphopyridine nucleotide (NADPH), uridine-5’-diphosphoglucuronic acid (UDPGA), saccharic acid-1, 4-lactone, and alamethicin were purchased from Sigma Chemical Co. (USA). Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). The pure water used in the current study was prepared using the Milli-Q system (Millipore, Bedford, MA, USA). All other materials were of analytical grade or higher.
Standardized preparation and quality control of the *Rhizoma coptidis* extract. The *Rhizoma coptidis* extract was prepared by standardized operating procedures, according to a previously reported method (Ma et al., 2010). The yield was 37.1%. The active compounds were determined by HPLC for quality control of the extract. The mass percent of berberine, coptisine, palmatine, and jatrorrhizine in the extract was 15.5%, 4.2%, 1.9%, and 3.2%, respectively.

**Synthesis of berberrubine and demethylenberberine.** To obtain berberrubine, berberine chloride (0.5 g) was heated at 200 °C for 20 min under vacuum (20-30 mmHg). The produced solid was re-crystallized with anhydrous ethanol for three times. After that, the product was treated with ethanol/concentrated hydrochloric acid (95:5) to get purified berberrubine (yield 60%). To obtain demethylenberberine, berberine chloride (0.5 g) and phloroglucin (0.5 g) were mixed with 60% H₂SO₄ (10 mL). Then the mixture was heated at 100 °C for 8 h. After that, the mixture was cooled in brine to get precipitate. The precipitate was then purified by high preparative performance liquid chromatography to obtain demethylenberberine (yield 26%). After synthesis of berberrubine and demethylenberberine, liquid chromatography (LC)-mass spectrometry (MS) and proton nuclear magnetic resonance spectroscopy (¹H NMR) were used to confirm their structural features.

**Animals.** Sprague-Dawley rats (Grade II) weighing 240–260 g were purchased from the Shanghai Slac Laboratory Animal Co. (Shanghai, China). The rats were housed in an air conditioned room at 22–24 °C under a 12 h dark/light cycle, and given food and water *ad libitum*. All animal experiments were performed in accordance with the guidelines of the National Research Council.
Liver microsome preparation. Liver microsomes were prepared as described (Hill, 2003), except the microsomes were resuspended and homogenized in ice-cold sucrose solution (0.25 M). Protein concentration was determined by BCA assay. The protein concentration was adjusted to 10 mg/mL using the sucrose solution, and then 0.5 mL aliquots were dispensed into labeled tubes and stored at –80 °C.

Intestinal S9 preparation. Intestinal cellular S9 fractions were prepared as described (Masaki et al., 2007), with modifications. Briefly, the small intestines were excised and rinsed with chilled 1.15% KCl. They were then cut into longitudinal strips. Mucosa was scraped and homogenized with 4 volumes of 50 mM ice-cold HEPES buffer (pH 7.4) containing 1.15% KCl. The homogenates were then centrifuged at 9000 g for 20 min at 4 °C. The protein concentrations were determined by BCA assay. The protein concentration was adjusted to 10 mg/mL, and then 0.5 mL aliquots were dispensed into labeled tubes and stored at –80 °C.

Identification of the metabolites. The experiments were performed on an LCQ ion-trap mass spectrometer (Thermo-Finnigan Corp., San Jose, CA, USA) with an ESI ion source in positive-ion detection mode. Samples were eluted through a reversed-phase column [Thermo Hypersil-Keystone C18, (5 µm, 2.1 × 150 mm)] at 25 °C with a linear gradient of A (0.1% formic acid and 2 mM ammonium formate) and B (methanol) (0 min, 80: 20; 3 min, 80: 20; 18 min, 52:48; 27 min, 5:95; 28 min, 80:20; 35 min, 80:20 ) at a flow rate of 0.3 mL/min. During the analysis, the ESI parameters were set as follows: sheath gas flow rate (arb): 30; aux/ sweep gas flow rate (arb): 10; source spray voltage (kv): 5; capillary temp (°C): 300; capillary voltage (v): 5; tube lens offset (v): 10. The MS^n product-ion spectra were produced by collision induced dissociation (CID) of the molecular ion of all analytes at their respective
HPLC retention times. Data acquisition was performed in full-scan LC/MS and MS/MS modes.

**HPLC-UV analysis of the metabolites.** A validated bio-analytical method based on HPLC was used to detect the metabolites of berberine; the HPLC was equipped with a 2695 separation module, a 2487 dual λ absorbance detector, and an Empower2 chemstation (Waters, USA). Separation and determination were performed using an XTerra RP 18 analytical column (5 µm, 3.9 × 150 mm) at 35 °C with λ = 346 nm. The samples were eluted through the column with a gradient of water-formic acid-triethylamine (100:0.1:0.2, v/v/v) and methanol (0 min, 75:25; 15 min, 60:40; 15.5 min, 75:25; 20 min, 75:25) at a flow rate of 0.5 mL/min. The standard sets had good linearity within their own quantitative range [0.078-2.5 µM for both metabolites (berberrubine and demethyleneberberine)]. The quality control samples were prepared at three different concentrations (0.156, 0.625, 2.25 µM).

**In vitro metabolism incubations.** Both phase I and II liver microsomal or intestinal S9 incubations were performed as described (Liu et al., 2009), with modifications. Briefly, reaction conditions including the protein concentration and the incubation time were optimized to ensure the linearity of the reactions. For the phase I microsomal or intestinal S9 incubation, the reactions were conducted in a medium containing 100 mM potassium phosphate buffer (pH 7.4) with 1 mM NADPH, 0.5 mg microsomal protein or 2 mg intestinal S9, and 10 or 50 µM berberine for liver microsomes or intestinal S9, respectively. After pre-incubation for 5 min, the reaction was initiated by the addition of the NADPH solution. After incubation at 37 °C for 15 min for liver microsomes or 60 min for intestinal S9, the reactions were terminated by adding an equal volume of cold methanol with internal standard.
For the phase II liver microsomal or intestinal S9 incubation, the reactions were conducted in a medium containing 0.5 mg microsomal protein or intestinal S9, 1 mM UDPGA, 10 mM MgCl₂, 5 mM saccharic acid-1,4-lactone, 50 mM potassium phosphate buffer (pH 7.4), and 10 or 15 μM demethyleneberberine for liver microsomes or intestinal S9, respectively. The microsomes were pretreated with 25 μg/mL alamethicin on ice for 15 min. After pre-incubation for an additional 5 min at 37 °C, the reactions were initiated by adding the UDPGA solution. After 2 min, the reactions were terminated by the addition of an equal volume of cold methanol with internal standard. After centrifugation at 12,000 rpm for 15 min at 4 °C, the samples were analyzed using the validated HPLC method. The semi-quantitative concentration of the phase II glucuronide-conjugated metabolite was calculated according to the accompanying calibration curve of demethyleneberberine. Control incubations with inactivated microsomes or intestinal S9 (prepared by incubation at 90°C for 5 min) or without NADPH/UDPGA were performed in parallel. All reactions were carried out in triplicate.

Influence of LPS on the metabolism of berberine in rat liver microsomes and intestinal S9. Twelve rats were used to study the influence of the LPS pretreatment on the metabolism of berberine in rat liver microsomes, while twenty rats were used in experiments performed on rat intestinal S9. In each experiment, the rats were randomly divided into two groups (male: female = 1: 1). One group of rats received an i.p. injection of 1 mg/kg LPS, the other received the same volume of saline. After pretreatment for 16 h, livers or intestines were excised, and then liver microsomes or intestinal S9 were immediately prepared and stored at -80 °C for in vitro metabolism experiments.

LC/MS/MS analysis of vinblastine. A Shimadzu Prominence UFLCXR series HPLC
and a Thermo Scientific TSQ Quantum Ultra mass spectrometer (Thermo, USA) equipped
with an electrospray ionization (ESI) source were used. After 40 µL 10% trichloroacetic acid
and 10 µL vincristine (500 ng/mL, internal standard) were added to 150 µL samples, the
mixture was extracted with 2 mL ethyl acetate. The supernatant was evaporated to dryness
under a stream of nitrogen. The residue was resolved with 150 µL mobile phase (50% water
phase and 50% organic phase). After being centrifuged at 16000 rpm for 5 min at 4 °C, 10 µL
samples were injected into the LC/MS/MS system. The samples were eluted through a
Hypersil Gold (C18) analytical column (3 µm, 100 × 2.1 mm) with a gradient of the aqueous
phase (0.2% v/v formic acid) and the methanol phase (0.2% v/v formic acid) (0 min, 80:20; 3
min, 5:95; 4 min, 5:95; 6 min, 80:20; 9 min, 80:20) at a flow rate of 0.3 mL/min. The ESI
source was set to positive ion mode. Data acquisition was performed in the multiple reaction
monitoring mode of the selective mass transition for each compound. The transitions of the
protonated precursor ions to the selected product ions were \( m/z 413.252 \rightarrow m/z 362.300 \) for
vincristine; \( m/z 406.203 \rightarrow m/z 271.700 \) for vinblastine. The quantitative ranges for measuring
vinblastine were 0.78-100 nM. The quality control samples were prepared at three different
concentrations (2, 15, 90 nM). The accuracy, precision, recovery and stability tests all met the
requirements of the quantitative determination in biological samples.

**LC/MS/MS analysis of the *Rhizoma coptidis* alkaloids.** A validated bio-analytical
method was used based on LC/MS/MS. An Agilent 1200 series HPLC and a triple-quadrupole
mass spectrometer (ABI4000, AB, USA) equipped with an electrospray ionization (ESI)
source were used. The samples were precipitated with three volumes of acetonitrile, with
phenacetin as internal standard. The supernatant was then mixed with an equal volume of
pure water, and then 10 μL samples were injected into the LC/MS/MS system. The samples were eluted through the XTerra RP 18 analytical column at 40 °C in aqueous phase (0.08% v/v formic acid in 4 mM ammonium acetate) and the acetonitrile phase (48:52) at a flow rate of 0.6 mL/min. The ESI source was set to positive ion mode. Data acquisition was performed in the multiple reaction monitoring mode of the selective mass transition for each compound. The transitions of the protonated precursor ions to the selected product ions were $m/z$ 338.10 → $m/z$ 322.90 for jatrorrhizine; $m/z$ 320.20 → $m/z$ 292.00 for coptisine; $m/z$ 336.10 → $m/z$ 292.00 for berberine; $m/z$ 352.00 → $m/z$ 335.90 for palmatine; and $m/z$ 180.00 → $m/z$ 110.00 for phenacetin. The quantitative ranges were 0.625–500 ng/mL for all measured Rhizoma coptidis alkaloids (berberine, coptisine, palmatine, and jatrorrhizine) in the tested biological samples. The quality control samples were prepared at three different concentrations (1.5, 25, 450 ng/mL). The accuracy, precision, recovery and stability tests all met the requirements of the quantitative determination in the biological samples.

**Studies on everted gut sac.** The transport of Rh-123, vinblastine, FD-4, and the Rhizoma coptidis alkaloids across the rat everted sac were evaluated as described (Veau et al., 2001), with modifications. Briefly, twelve rats were used in each everted gut sac experiment. In each experiment, the rats were randomly divided into two groups (male: female = 1: 1). One group of rats received an i.p. injection of 1 mg/kg LPS, the other received the same volume of saline. After 16 h, the rats were sacrificed and the ileum was removed 5 cm above the caecum after laparotomy. The ileum was washed with chilled saline and everted. A 12 cm segment was cut and ligated at one end. As for the study of the absorption of the Rhizoma coptidis alkaloids or FD-4, the everted gut sac was filled in the serosal side (inside) with 1
mL blank Krebs-Ringer buffer (containing 118 mM NaCl, 25 mM NaHCO₃, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 11 mM glucose, 1.2 mM KH₂PO₄, and 4.7 mM KCl, pH 6.8). The other end was tightly ligated to create a gut sac. The sac was immediately incubated in a Magnus bath containing 19 mL oxygenated Krebs-Ringer buffer at 37 °C. After 5 min, 1 mL *Rhizoma coptidis* extract (1 mg/mL in Krebs-Ringer buffer) or FD-4 solution (2 mg/mL in Krebs-Ringer buffer) was added to the bath. Aliquots (200 μL) were taken from the serosal side every 15 to 60 min. The same volume of blank Krebs-Ringer buffer was resupplied each time. With regard to the study of the efflux of Rh-123, vinblastine, or berberine across the everted sac, the sac was filled on the serosal side with 1 mL Rh-123 (250 μM in Krebs-Ringer buffer), vinblastine (10 μM, dissolved in DMSO and then diluted with Krebs-Ringer buffer, the final concentration of DMSO was less than 1‰) or berberine solution (1, 3, and 10 μg/mL, dissolved in methanol and then diluted with Krebs-Ringer buffer, the final concentration of methanol was less than 1‰), and tightly ligated to create a gut sac. The gut sac was immediately incubated in a Magnus bath containing 20 mL oxygenated Krebs-Ringer buffer at 37 °C. Aliquots (200 μL) were taken from the mucosal side (outside) every 15 to 60 min. The same volume of blank Krebs-Ringer buffer was resupplied each time. After incubation was terminated, the lengths of the sacs were measured. The concentrations of Rh-123 and FD-4 were determined using a fluorescence microplate reader (BioTek Instruments, Inc, Winooski, USA) with excitation at 485 nm and emission at 528 nm. The concentrations of vinblastine and the *Rhizoma coptidis* alkaloids were determined using LC/MS/MS.

**Time-dependent influence of LPS pretreatment on the pharmacokinetics of berberine in rats received *Rhizoma coptidis* extract.** Twelve male rats were randomly
divided into four groups. Three groups of rats received an i.p. injection of 1 mg/kg LPS, one group of rats received the same volume of i.p. injected saline as control. After 16, 24, or 36 h of the LPS pretreatment, the rats were administered 0.72 g/kg the oral extract of *Rhizoma coptidis* (the dosages of berberine was 111.6 mg/kg). The rats in the control group were also administered the oral extract. Venous blood samples (about 0.3 mL) were collected in heparinized 1.5 mL tubes at 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h after *Rhizoma coptidis* ingestion. Blood samples were centrifuged at 12,000 rpm for 10 min immediately upon collection, and the plasma was collected and stored at −80°C for later analysis using the validated LC/MS/MS method.

**Influence of LPS pretreatment on the pharmacokinetics of the *Rhizoma coptidis* alkaloids in rats.** Twelve rats were randomly divided into two groups (male: female = 1: 1). One group of rats received an i.p. injection of 1 mg/kg LPS, the other received the same volume of saline. After 16 h of pretreatment, the rats were administered 0.72 g/kg oral extract of *Rhizoma coptidis* (the dosages of berberine, coptisine, palmatine, and jatrorrhizine were 111.6, 30.2, 13.7, and 23.0 mg/kg, respectively). As described above, venous blood samples were collected, and then the plasma was prepared and stored at −80°C for later analysis using the validated LC/MS/MS method.

**Histopathological and ultrastructural examinations.** Histopathological and ultrastructural examinations were performed as described (Hang et al., 2003). For light microscopy, segments of the ileum were excised immediately after the rats were sacrificed. Tissues fixed in 4% formaldehyde were processed and stained with hematoxylin and eosin. For electron microscopy, segments of the ileum were fixed in 2% glutaraldehyde/cacodylate.
buffer (pH 7.4) and processed for routine transmission electron microscopy. Tissues were cut to obtain longitudinal sections of epithelial cells. Ultrathin sections were placed on copper grids stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope.

**Statistical analysis.** The area under the concentration versus time curve (AUC) was calculated according to the trapezoidal rule. The results were expressed as mean ± S.D. The Student’s t-test was used to compare a single treatment mean with a control mean. ANOVA was performed to compare multiple treatment means. When the analysis of variance manifested a statistical difference, the Dunnetts test was applied when needed. Significant differences of the results of the everted gut sac experiments were determined using two-factor analysis of variance by the general linear model analysis. $P<0.05$ was considered significant, and $P<0.01$ was considered highly significant.
Results

Time-dependent influence of LPS pretreatment on the pharmacokinetics of berberine in rats received Rhizoma coptidis extract. The systemic exposures (AUC$_{0-24\,\text{h}}$) to berberine in Rhizoma coptidis are shown in Figure 2. The result showed that the LPS pretreatment for 16 h significantly increased the AUC$_{0-24\,\text{h}}$ of berberine (P=0.024).

Influence of LPS on the pharmacokinetics of the Rhizoma coptidis alkaloids in rats. Both the mean plasma concentration-time curves and the systemic exposures (AUC$_{0-24\,\text{h}}$) to the alkaloids in Rhizoma coptidis are shown in Figure 3. The result showed that the LPS pretreatment significantly increased the AUC$_{0-24\,\text{h}}$ of berberine, coptisine, and jatrorrhizine (P=0.025, 0.03, and 0.006, respectively).

Influence of LPS on the metabolism of berberine in rat liver microsomes. Two major phase I metabolites (Fig.1), M1 (berberrubine) and M2 (demethyleneberberine), with fragment ions at $m/z$ 322 and $m/z$ 324 according to the LC/MS/MS identification, were formed during the incubation of berberine with rat liver microsomes in the presence of NADPH. The incubation of demethyleneberberine in the rat liver microsomes in the presence of UDPGA led to the formation of ((glucuronide of demethyleneberberine, Fig.1), with fragment ions at $m/z$ 500 according to the LC/MS/MS identification. As shown in Figure 4, the LPS pretreatment did not influence both the phase I metabolism of berberine (0.72 ± 0.24, and 0.70 ± 0.18 µM/min/mg protein for M1, and M2 formation in the control microsomes, respectively) and phase II metabolism of demethyleneberberine (39.2 ± 9.5 µM/min/mg
Influence of LPS on the metabolism of berberine in intestinal S9. Similar to that observed in the incubation in liver microsomes, both M1 and M2 were formed during the incubation of berberine in intestinal S9 in the presence of NADPH. However, the generated M1 was scarce. The incubation of demethyleneberberine in the presence of UDPGA led to the formation of M3. The results showed that the LPS pretreatment significantly decreased both the phase I metabolism of berberine to M2 (5.23 ± 2.66 nM/min/mg protein for M2 formation in control intestinal S9, P=0.006) and phase II metabolism of demethyleneberberine to M3 (19.5 ± 4.5 µM/min/mg protein for M3 formation in control intestinal S9, P=0.016) (Fig. 5).

Influence of LPS on the absorption of the *Rhizoma coptidis* alkaloids in rat everted gut sacs. The mean absorption amount-time curves of the *Rhizoma coptidis* alkaloids are shown in Figure 6. The result showed that the LPS pretreatment significantly increased the absorption of the *Rhizoma coptidis* alkaloids (P=0.036, 0.032, 0.005, 0.003 for berberine, coptisine, jatrorrhizine, and palmatine, respectively.).

Influence of LPS on the efflux of berberine, vinblastine and Rh-123 in rat everted gut sacs. The result showed that there was no significant difference between the efflux ratios of berberine at different concentrations. The mean efflux ratio-time curves of berberine are shown in Figure 7(A). The results showed that the LPS pretreatment significantly decreased the efflux of berberine (P=0.014). The efflux ratio of vinblastine (P=0.003) as well as Rh-123 (P=0.021) was also significantly decreased [Fig. 7(B) & (C)].

Influence of LPS on the absorption of FD-4 in rat everted gut sacs. As shown in Fig. 8, the LPS pretreatment had no significant influence on the absorption of FD-4.
Influence of LPS on mucosal structure. Compared to normal mucosa [Fig. 9(A)], the LPS pretreatment caused significant damages to mucosal structure [Fig. 9(B)]. The villi became shorter and blunt. The adjacent villi fused into one piece. Some epithelial cells were shed from the top of the villi. Obvious ultrastructural alterations [Fig. 9(D)], including sparse and ruptured microvilli, swollen mitochondria and endoplasmic reticulum, and apoptotic bodies in the nuclei, were also found when compared to the normal control [Fig. 9(C)]. However, the tight junction between epithelial cells remained intact.
Discussion and conclusions

Systemic exposure to LPS leads to systemic inflammation or endotoxemia. Generally speaking, LPS in bloodstream could be derived from two sources, one is the contamination of tissues, fluids or foreign bodies, the other is bacterial and endotoxin translocation from the intestinal lumen (Hauser et al., 2011). However, although the gastrointestinal tract contains a very high intraluminal concentration of living bacteria, the gut barrier restricts micromolecular permeation and almost completely restricts macromolecular permeation in normal subjects and experimental animals (Balzan et al., 2007). Hence, intestinal bacteria and endotoxins translocation do not normally occur in the healthy adult subjects and experimental animals. Therefore, although it is looked as a very severe form of infection comparable to a bacterial septicemia, i.p-injection of LPS was still usually used to induce systemic inflammation or endotoxemia in experimental animals (Morgan et al., 2008). On the other hand, several diseases, where *Rhizoma coptidis* is useful therapy, were associated with endotoxemia, including ulcerative colitis (Amati et al., 2003), acute gastrointestinal syndrome (Zhang et al., 2010), cardiac-related diseases (Mou et al., 2002), diabetes (Pussinen et al., 2011), hepatic fibrogenesis (Lin et al., 2011). Therefore, the LPS model was used in this study.

The results showed that increased systemic exposure to the four *Rhizoma coptidis* alkaloids in rats that received an oral administration of *Rhizoma coptidis* extract after LPS pretreatment, indicating that the bioavailability of the *Rhizoma coptidis* alkaloids improved in
LPS pretreated rats. These results add to the understanding of why *Rhizoma coptidis* is curative in inflammation-related pathological conditions despite the low plasma concentrations of the alkaloids measured in normal subjects and experiment animals. Furthermore, we reported recently that the LPS pretreatment increased the acute toxicity of the *Rhizoma coptidis* extract in mice (Ma et al., 2011). Certain classical works of TCM, such as *Ben Cao Yan Yi (Augmented Materia Medica)*, have stated that although the *Rhizoma coptidis* extract is an excellent therapeutic agent for dysentery, the dosage should be adjusted based on the severity of the disease; otherwise, the health of the patient would be endangered. On the other hand, berberine, the main toxic constituent of the *Rhizoma coptidis* extract (Ma et al., 2010), has caused respiratory failure, extrapyramidal system reactions, severe arrhythmia, liver function injury, and even death in clinics in China (Li et al., 2008). These reports together strongly suggested that the *Rhizoma coptidis* extract as well as its alkaloids was not only curative but also potential toxic, and should be carefully used in clinics, especially in LPS-related inflammatory diseases.

We conclude that LPS increased the systemic exposure of the *Rhizoma coptidis* alkaloids through the enhancement of their absorption on the basis of the following results: (1) the *in vitro* absorption of the *Rhizoma coptidis* alkaloids increased; (2) both the *in vitro* phase I metabolism of berberine and phase II glucuronidation metabolism of demethyleneberberine were non-significantly influenced in the liver microsomes.

Given that the *Rhizoma coptidis* alkaloids are substrates of P-gp (Zhang et al., 2011), the reduced intestinal efflux of berberine indicated that the function of the intestinal P-gp was decreased by LPS pretreatment. This assumption is verified by the reduced intestinal efflux of
vinblastine and Rh-123, substrates of P-gp (Ogihara et al., 2006; Tomita et al., 2010). Because P-gp is mainly expressed on the brush border membrane of epithelial cells (Panwala et al., 1998), damages to mucosal structure, such as rupture of the microvilli and apoptosis of the epithelial cells, may be involved in the decreased function of P-gp caused by LPS pretreatment. Conversely, the intact tight junction and unaffected absorption of FD-4 exclude the possibility that the increased absorption of the *Rhizoma coptidis* alkaloids is due to paracellular transportation. On the other hand, both the *in vitro* phase I metabolism of berberine and phase II glucuronidation metabolism of demethyleneberberine were significantly decreased in intestinal S9. Given that intestinal metabolism participates in the low plasma levels of the *Rhizoma coptidis* alkaloids (Liu et al., 2010), the results suggest that the increased intestinal absorption of the *Rhizoma coptidis* alkaloids was due to not only the decreased intestinal efflux but also the diminished intestinal metabolism.

The metabolites formed during the incubation of berberine and demethyleneberberine were consistent with a previous report (Liu et al., 2009). In the *in vitro* phase II metabolism experiments, only the incubation of the M2 standard was tested. Both M1 and M2 were reported to be mainly catalyzed by UGT1A1 (Liu et al., 2009), suggesting that the incubation of the M2 standard reflects the metabolic capability of UGT1A1. The results of the *in vitro* metabolism showed the different influences of LPS on hepatic and intestinal drug-metabolizing enzymes. LPS pretreatment has been reported to show different influences on the expression of CYPs in the liver and intestine (Chung et al., 2008), which may be related to the tissue-specific concentrations of the effectors and the severity of pathological changes. The absence of hepatic metabolism modulation of LPS in the current work may have
been caused by the different experimental settings, especially the initiation time of the experiment after LPS administration. LPS pretreatment has been reported to impose time-dependent (Yang and Lee, 2008) and biphasic effects on some hepatic P450 catalytic activities (Sewer and Morgan, 1998). For example, the hydroxylation of testosterone at the 2α, 16α, and 6β positions was decreased 6 h after LPS pretreatment, and then returned to normal by 12 h before decreasing again at 24 h (Sewer and Morgan, 1998). Most of the changes in the enzyme activities in rat liver were observed beyond 24 h after LPS pretreatment (Yang and Lee, 2008). According to the preliminary study, the LPS pretreatment for 16 h showed the strongest influence on the systemic exposure of berberine, the major *Rhizoma coptidis* alkaloid. Therefore, we only studied the influences of the pretreatment of LPS for 16 h in both the *in vivo* and *in situ* experiments. Hence, the absence of the hepatic metabolism modulation of LPS in this study was not a surprising outcome.

In conclusion, our results showed that LPS pretreatment increased systemic exposure to the *Rhizoma coptidis* alkaloids in rats that were orally administered with *Rhizoma coptidis* extract. This increase was due to the enhancement of the intestinal absorption of the alkaloids, which was related to both the decreased intestinal efflux and intestinal metabolism.
Authorship Contributions


Contributed new reagents or analytic tools: F.-R.Q., and C.-H. W.

Performed data analysis: Y.-M.M. and B.-L.M.

Wrote or contributed to the writing of the manuscript: B.-L.M. and Y.-M.M.
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Footnotes

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Legend for Figures

Fig. 1. Structures of four *Rhizoma coptidis* alkaloids (berberine, coptisine, palmatine, and jatrorrhizine) and three metabolites of berberine.

Fig. 2. Time-dependent effects of LPS pretreatment (1 mg/kg) on the pharmacokinetics of berberine in rats received an oral *Rhizoma coptidis* extract (mean ± S.D., n=3). Pre-16, Pre-24, Pre-36: the LPS pretreatment for 16, 24, and 36 h, respectively. *, $P<0.05$ compared with the control.

Fig. 3. Effect of LPS (1 mg/kg) pretreatment on the pharmacokinetics of the *Rhizoma coptidis* alkaloids in rats orally administered with *Rhizoma coptidis* (0.72 g/kg) (mean ± S.D., n=6). (A) Effect of LPS pretreatment on the mean plasma concentration-time curves of the *Rhizoma coptidis* alkaloids. (B) Effect of LPS pretreatment on the AUC$_{0-24\text{ h}}$ (area under concentration-time curve from 0 to 24 h) of the *Rhizoma coptidis* alkaloids.
Ber: berberine; Cop: coptisine; Pal: palmatine; Jat: jatrorrhizine. *, $P<0.05$, **, $P<0.01$ compared with the control (*Rhizoma coptidis*).

Fig. 4. Effect of LPS (1 mg/kg) pretreatment on the phase I metabolism of berberine (metabolites: M1 and M2) and phase II metabolism of demethyleneberberine (metabolite: M3) in rat liver microsomes (mean ± S.D., n=6).
M1 (berberrubine), M2 (demethyleneberberine), and M3 (glucuronide of demethyleneberberine): metabolites at m/z 322, 324, and 500, respectively.

Fig. 5. Effect of LPS (1 mg/kg) pretreatment on the phase I metabolism of berberine (metabolite: M2) and phase II metabolism of demethyleneberberine (metabolite: M3) in rat intestinal S9 (mean ± S.D., n=10).

M2 (demethyleneberberine) and M3 (glucuronide of demethyleneberberine): metabolites at m/z 324, and 500, respectively. *, P<0.05, **, P<0.01 compared with the control.

Fig. 6. Effect of LPS (1 mg/kg) pretreatment on the absorption of the Rhizoma coptidis alkaloids in rat everted gut sac (mean ± S.D., n=6).

Ber: berberine; Cop: coptisine; Pal: palmatine; Jat: jatrorrhizine.

Fig. 7. Effect of LPS (1 mg/kg) pretreatment on the efflux ratios of berberine (A), vinblastine (B), and Rh-123 (C) in rat everted gut sac (mean ± S.D., n=3 for berberine, n= 6 for vinblastine, and Rh-123).

Fig. 8. Effect of LPS (1 mg/kg) pretreatment on the transport of fluorescein isothiocyanate-labeled dextran (FD-4, average molecular mass 4 kDa) in rat everted gut sac (mean ± S.D., n=6).

Fig. 9. Effect of LPS (1 mg/kg) pretreatment on mucosal structure. (A) Histopathological
examination of the control group; (B) histopathological examination of the LPS pretreatment group, damages are denoted by the arrows; (C) ultrastructural examination of the control group; (D) ultrastructural examination of the LPS pretreatment group, damages are denoted by the arrows. For (A) and (B), original magnification: ×200; for (C) and (D), original magnification: ×16500.
Fig. 1

Coptisine

Jatrorrhizine

Palmatine

Berberine

M2 (m/z 324)

M1 (m/z 322)

M3 (m/z 500)
Fig. 2

The bar chart shows the AUC0-24 h (% of control) for different conditions: Control, Pre-16, Pre-24, and Pre-36. The y-axis represents the AUC0-24 h (% of control) ranging from 0 to 250. The x-axis represents the conditions. Pre-16 shows a significant increase compared to the control group, indicated by the asterisk. The error bars indicate the variability of the data.
Fig. 3

A

Concentration (ng/mL)

Time (h)

Δ *Rhizoma Coptidis*  
▽ *Rhizoma Coptidis + LPS*

AUC$_{0-24h}$ (ng · h/mL)

Time (h)

[Graph showing concentration over time for different treatments and AUC values for each treatment group (Jat, Cop, Pal, Ber)]
Fig. 4

Formation of metabolites (% of control)

M1  M2  M3
Fig. 5

Formation of metabolites (% of control)

M2

M3

* *

*
Fig. 8

Trans-membrane transport ratio of FD-4 (%/cm sac)

- △ Control
- ▼ LPS

Time (min)

0 15 30 45 60