Combined contribution of increased intestinal permeability and inhibited deglycosylation of ginsenoside Rb1 in intestinal tract to the enhancement of ginsenoside Rb1 exposure in diabetic rats following oral administration

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Abbreviations

AUC, area under the concentration-time curve; C-K, compound-K; CYP450 cytochrome P450; DM, diabetes mellitus; FD4, fluorescein isothiocyanate-dextran of 4 kDa; FLU, fluorescein sodium; HFD, high-fat diet; HBSS, hanks’ balanced salt solution; LC-MS, liquid chromatography–mass spectrometry; Oatp, organic anion-transporting polypeptide; P_app, apparent permeability coefficient; P_eff, apparent effective permeability; P-gp, P-glycoprotein; PPD, protopanaxadiol; Rb1, ginsenoside Rb1; STZ, streptozotocin; TEER, transepithelial electrical resistance.
Abstract

Panax ginseng is becoming a promising anti-diabetic herbal medication. As the main active constituents of Panax ginseng, ginsenosides are well-known poorly absorbed chemicals. However, the pharmacokinetic behavior of ginsenosides under diabetic condition is not fully understood. The aim of this study was to explore the alternations and potential mechanisms of pharmacokinetic behavior of ginsenoside Rb1 (Rb1) in diabetic (DM) rats compared with that in normal (CON) rats and high-fat diet (HFD) rats. Systemic exposure ($\text{AUC}_{0-\infty}$) of Rb1 was significantly raised in diabetic rats following oral administration. Oral bioavailability ($\text{F\%}$) of Rb1 was significantly higher in diabetic rats (2.25%), compared to CON (0.90%) and HFD (0.78%) rats. Further studies revealed that increased Rb1 exposure in diabetic rats may be mainly attributed to increased Rb1 absorption via intestine and inhibited Rb1 deglycosylation by intestinal microflora. Neither metabolic enzymes nor drug transporters displayed appreciable effects on Rb1 disposition. The transport of paracellular markers (FLU and FD-4) as well as Rb1 itself across Caco-2 monolayer cultured with diabetic serum was promoted, demonstrating that increased paracellular permeability of Caco-2 monolayer may benefit intestinal Rb1 absorption. Additionally, Rb1 exposure was decreased in diabetic rats following Rb1 intravenous administration, which may result from the increased Rb1 urinary
excretion. In conclusion, Rb1 oral exposure was significantly increased under diabetic condition, which is of positive significance to clinical treatment. The potential mechanism may be associated with combined contribution of increased gut permeability and inhibited deglycosylation of ginsenoside Rb1 by intestinal microflora.
Introduction

Panax ginseng (*Panax ginseng C.A. Meyer*) is becoming a popular remedy and dietary supplement which has a broad range of pharmacological actions (Attele et al. 1999). Ginsenosides are considered to be the main effective constituents of Panax ginseng, among which ginsenoside Rb1 (Rb1) is one of the most abundant components. Accumulating evidences have showed that ginsenosides including Rb1 possesses anti-diabetic properties (Kimura et al. 1981; Sotaniemi 1995; Vuksan et al. 2000; Attele et al. 2002; Xie et al. 2005; Liu et al. 2014). However, oral bioavailability of Rb1, Rg1 and Rh2 were reported to be only 0.1%, 2% and 5%, respectively (Odani et al. 1983a,b). As a result, it remains to be clarified how low plasma concentration of ginsenosides exert glucose-lowering action.

Accumulating reports demonstrated that diabetes may influence pharmacokinetics of numerous drugs. For example, faster clearance and lower AUC of telithromycin and clarithromycin were shown in diabetic rats (Lee et al. 2008; Kim et al. 2005) while diabetes induced increased plasma concentration of verapamil after oral administration (Hu et al. 2011). Diabetes not only affects protein, lipids and carbohydrate metabolism but also regulate gastric emptying, non-enzymatic glycation of albumin, renal function, metabolic enzymes and transporters which are all involved in drug pharmacokinetics (Dostalek et al. 2012). Alterations
of activity and expression of Cytochrome P450s and drug transporters by diabetes were mostly considered as the potential underlying mechanism. We previously reported that diabetes oppositely altered pharmacokinetic behaviors of verapamil following oral and intravenous administration to rats via oppositely regulating the activity and expression of hepatic and intestinal CYP3A respectively (Hu et al. 2011). The exposure of orally administered glibenclamide induced by diabetes was increased which attributed to both impaired hepatic CYP2C11 and intestinal BCRP activity and expression (Liu et al. 2012). Similarly, the exposure of berberine following oral dose was increased in diabetic rats due to down-regulated intestinal P-glycoprotein (P-gp) (Yu et al. 2010). In addition, intestinal mucosa with intact tight junctions also serves as a main barrier to the passage of some drugs. For most of ginsenosides, large molecular mass (>500 Da), high hydrogen bond counts and high molecular flexibility were responsible for their low membrane permeability (Liu et al. 2009). It is observed that diabetic patients displayed high serum zonulin levels, associated with the increased intestinal permeability (Bosi et al. 2006; Sapone et al. 2006). These facts indicated that pharmacokinetic behaviors of ginsenosides may be greatly changed on account of the alteration of CYP450s, transporters or intestinal permeability under diabetic condition.

It is generally accepted that ginsenosides are extensively degraded
by intestinal microflora in the gastrointestinal tract (Hasegawa et al. 1996; Tawab et al. 2003; Liu et al. 2009). Rb1, belonging to 20-\((S)\)-protopanaxadiol-type (PPD-type) saponins (Fig. 1A), can be transformed to ginsenoside Rd (Rd), ginsenoside Rg3 (Rg3), ginsenoside Rh (Rh), ginsenoside F2 (F2), compound-K (C-K) and 20\((S)\)-protopanaxadiol (Ppd) via stepwise cleavage of four sugar moieties at the C-3 and C-20 positions (Fig. 1B). The disorder of intestinal microflora was verified under diabetic condition, indicating that the alteration of biotransformation catalyzed by intestinal microflora may affect degradation of ginsenosides in intestinal gut, leading to changed pharmacokinetic properties of ginsenosides.

The present study was designed to investigate whether the pharmacokinetic profiles of Rb1 were altered in type 2 diabetic rats induced by a combination of high-fat diet and low-dose streptozotocin (STZ). The possible influential factors including alteration of CYP450s, transporters, gut permeability and deglycosylation metabolism induced by diabetes were taken into consideration. As a therapeutic agent for diabetes, a comprehensive study on pharmacokinetic characteristics of Rb1 under diabetic conditions is definitely worthwhile.
Materials and Methods

Chemicals

Ginsenoside Rb1, Rd, Rg3, F2, Rh2, C-K, 20(S)-protopanaxadiol (Ppd) (purity > 99%) were purchased from Jilin University (Changchun, China). Pentobarbital, digoxin, streptozotocin (STZ), verapamil, cyclosporine A, BSP, rifampicin, naringin, levofloxacin, fluorescein isothiocyanate-dextran of 4 kDa (FD-4) and fluorescein sodium (FLU) were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol and acetonitrile was obtained from Merck (Damstadt, Germany). All the other reagents were analytical grade and commercially available.

Animals

Male Sprague-Dawley rats (weighing 100-120 g) were purchased from Sino-British Sipper & BK Lab Animal Ltd. (Shanghai, China) and acclimated to the laboratory environment for 3 days. The rats were maintained in a controlled environment of temperature (23±1°C) and relative humidity (50%±5%) with 12 h light/darkness cycle. Water and food were provided ad libitum. All animal studies were conducted according to protocols approved by the Animal Ethic Committee of China Pharmaceutical University.
Induction of diabetic rats

Diabetic rats were induced as described previously by combination of high-fat diet and low-dose STZ injection (Chen et al. 2011, Liu et al. 2013). Briefly, rats were divided into three groups randomly: control (CON) rats, high-fat diet fed (HFD) rats and diabetic (DM) rats. CON rats were fed on normal chow while both HFD rats and DM rats were fed on high-fat diet (Xietong biotech, Jiangsu, China), which consisted of 15% lard, 5% sesame oil, 20% sucrose, 2.5% cholesterol and 57.5% normal chow. After four-week of dietary manipulation, DM rats received an intraperitoneal injection of STZ (35 mg/kg, dissolved in citrate buffer, pH 4.5). Both HFD and CON rats received only citrate buffer. Subsequently, the experimental rats maintained their original diets. On day 7 post-STZ injection, rats with the fasting blood glucose levels exceeded 11.1 mM were considered to be diabetic. The following experiments were performed on the 28th day after STZ injection.

Pharmacokinetics of Rb1 following oral and intravenous administration

For oral administration, rats were fasted overnight and followed by received an oral dose of Rb1 (100 mg/kg). Blood samples were collected under light ether anesthesia via the orbital sinus at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h post oral dose. For intravenous administration,
Rb1 (10 mg/kg) were given to rats via tail vein. Blood samples were collected at 0.167, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 48 and 72 h after intravenous administration. After 3 or 4 samplings, the appropriate amount of normal saline was administered to the rats to compensate for blood loss. Plasma samples were obtained by centrifugation at 4000 rpm for 10 min and stored at −80°C until analysis.

Portal plasma samples were also collected from another subset of experimental rats. The fasted rats were anesthetized using pentobarbital (60 mg/kg, i.p.) and portal vein cannula was performed. Portal blood samples were collected via cannula at 1, 2, 4, 6 and 8 h following oral administration of Rb1 (100 mg/kg).

**Rb1 absorption via intestinal wall**

Rb1 absorption via intestinal walls was evaluated by *in situ* single-pass perfusion as described previously (Yu et al., 2010). In brief, fasted rats were anesthetized using pentobarbital (60 mg/kg, i.p.) and followed by inserted two cannulas for input and output at the two ends of isolated jejunum (10 cm). The jejunum was returned to the abdominal cavity and the abdomen was closed. The isolated jejunal segment was pre-perfused with 0.9% saline solution (37°C) at 0.2 mL/min for 20 min, followed by Krebs-Henseleit buffer containing Rb1 (2 μg/mL) and phenol red (5 μg/mL for impermeable volume marker). After a steady state was
achieved (30 min), consecutive effluent samples were collected at 15-min intervals through the distal cannula for 120 min. At the end of the experiments, the animals were killed, perfused intestinal segments were removed, and the areas of absorption were measured. The apparent effective permeability ($P_{\text{eff}}$, cm/min) was calculated according to the following equation: $P_{\text{eff}} = -\frac{Q \ln(C_{\text{out}}/C_{\text{in}})}{A}$, where $C_{\text{out}}$ and $C_{\text{in}}$ indicate the output and input of Rb1 concentration, respectively. $A$ (cm$^2$) represents the area of the perfused intestinal segment, and $Q$ is the flow rate (0.2 mL/min).

**Rb1 excretion via urine and bile**

For urinary excretion, experimental rats were housed individually in metabolic cages prior to the study. After 3-day adaption, rats were intravenously received Rb1 (10 mg/kg). Urine samples were collected before dosing and at intervals of 0-6 h, 6-12 h, 12-18 h, 18-24 h, 24-36 h, 36-48 h and 48-72 h after dosing. For biliary excretion, rats were anesthetized using ether and biliary cannula was applied via the common bile duct. After confirmation of bile flow, Rb1 (10 mg/kg) was intravenously administered to the rats and bile samples were collected before dosing and at intervals of 0-1 h, 1-2 h, 2-3 h, 3-4 h, 4-6 h and 6-8 h after dosing. Aliquots of urine and bile samples were stored at −80°C until analysis.
Rb1 metabolism in rat hepatic and intestinal microsome

Rat hepatic and intestinal microsome were prepared freshly, according to previously described method (Xie et al., 2010). Rb1 metabolism was determined by measuring the depletion of Rb1. Incubation system containing rat hepatic or intestinal microsome (2 mg/mL), Rb1 (0.2 μM or 0.05 μM of final concentration for hepatic or intestinal microsome respectively) in 0.1 M phosphate-buffered saline (pH 7.4). The incubation mixture was pre-incubated for 5 min at 37 °C, and reaction was initiated with the addition of an NADPH-regenerating system (0.5 mM NADP, 10 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, and 5 mM MgCl2). After the designated time (0, 30, 60, 120 min), the reaction was terminated by adding 1 mL of iced water-saturated n-butanol. All incubations were performed in triplicate.

Rb1 metabolism in rat intestinal content

The rats were sacrificed under ether anesthesia, the fresh contents of small intestine and large intestine were quickly harvested, respectively. The intestinal contents were homogenized with anaerobic medium in the ratio of 1 g : 5 mL under anaerobic environment immediately, filtrated with gauze and centrifugated at 825 g for 10 min at 4°C. The fresh
cultural solution (supernatants) was collected in anaerobic incubation bags and used for assessing Rb1 metabolism. Rb1 (final concentration 0.65 mg/mL) was added into above fresh cultural solution and incubated at 37°C in shaking water-bath for 0.5, 1, 2, 4, 6, 8 and 12 h. The reaction were terminated by cooling down to 4 °C at the designed times. Contents of Rb1 and its metabolites in reacting systems were determined by LC-MS method.

**Rb1 uptake and transport across cell monolayer**

Caco-2 cells and MDR1-MDCK cells were cultured in DMEM containing 10% FBS, 1% NEAA, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO₂ at 37 °C. Wild-type MDCK cells were also used as control.

Rb1 Bidirectional transport experiments were carried out in triplicate at 2 and 10 μM for Rb1 with or without inhibitors in Hanks’ balanced salt solution (HBSS). In brief, MDR1-MDCK cells were seeded in Millicell inserts (1.2 cm diameter, 0.4 μm pore size; Millipore, USA) and cultured. The integrity of the cell layer was monitored by measurement of transepithelial electrical resistance (TEER) with Millicell-ERS equipment (Millipore, USA). Only the monolayer with a TEER value of more than 300 Ω·cm² was used. Rb1 was loaded onto either apical (A) or basolateral (B) compartments with or without inhibitors. Samples were taken at 2 h
from the opposite compartment. The apparent permeability coefficient (P_{app}) was calculated as follows:

\[ P_{app} = \frac{dQ}{AC_0 dt} \]

Where \( dQ/dt \) is the rate of permeability (nmol/s); \( A \) is the surface area of the insert (cm\(^2\)); \( C_0 \) is the initial concentration.

Effect of experimental rat serum on Caco-2 monolayer permeability and Rb1 transport across Caco-2 monolayer were conducted. Transport studies were initiated in the same way as described above. Exceptionally, medium was replaced by DMEM containing 10% rat serum 8 days prior to the study in order to simulate \textit{in vivo} condition. The serum was collected from age-matched CON, HFD and DM rats and inactivated for 30 min at 56°C. The Caco-2 monolayer permeability was evaluated by measuring FD-4 and FLU transport (A to B) across monolayer. Meanwhile, Rb1 transport was also performed under the same condition.

Effect of transporter inhibitors on Rb1 uptake by Caco-2 cells was also measured. Caco-2 cells cultured in the 24-well plate were incubated with Rb1 (50 \( \mu \text{M} \)) with or without inhibitors in HBSS. Cellular uptake was terminated by removing the incubation solution and rinsing with iced HBSS for three times. Then, purified water was added to each incubated well, frozen and melted repeatedly three times and ultrasonically treated to break down cells. Rb1 was extracted by water-saturated \( n \)-butanol. Cellular Rb1 content was normalized to protein contents which were
determined using Bradford method.

**Determination of ginsenosides by LC-MS**

Validated LC-MS method was employed to analyze ginsenosides content, according to the method described before with minor modifications (Liu et al. 2013). Briefly, ginsenosides were extracted with water-saturated n-butanol from biological samples, including plasma, urine, bile, intestinal perfusate, microsome and cell culture medium. Separation was performed at a flow rate of 0.2 mL/min with a Waters Symmetry C18 column (5.0 μm, 2.1 mm×150 mm). The mobile phase was composed of a mixture of NH₄Cl (0.15 mM) in water (A) and acetonitrile (B). The gradient conditions were as follows: 0-3 min at 25% B, 3-5 min 25%→50% B, 5-14 min 50% B, 14-18 min 50%→65% B, 18-28 min at 65% B, 28-29 min 65→25%, 29-32 min 25%. Analysis in the mass spectrometer with ESI probe was operated in the selected ion monitoring model: m/z [M+Cl]²⁻ 589.25 for Rb1, [M+Cl]⁻ 981.45 for Rd, [M+Cl]⁻ 819.4 for Rg3 and F2, [M+Cl]⁻ 657.3 for Rh2 and C-K, [M+Cl]⁻ 495.25 for Ppd, [M+Cl]⁻ 815.35 for digoxin (internal standard). The injection volume was 5 μL. Calibration curves constructed for the analytes (10-1000 ng/mL) showed good linearity (r² > 0.999).
Statistical analysis

Pharmacokinetic parameters were calculated by non-compartmental analysis (Pheonix Winnonlin 6.1, Pharsight, St. Louis MO). The area under the concentration-time curve (AUC) was calculated by trapezoidal rule with extrapolation to infinity. The oral clearance (CL) was calculated as dose/AUC. The terminal elimination constant (k) was obtained from the least-square linear regression slope of ln-concentration versus time and terminal elimination half-life (T1/2) was calculated as 0.693/k. All data were expressed as mean ± S.E.M. Statistical differences among groups were evaluated using one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc test. A P value of less than 0.05 was considered to be statistically significant.

Results

Pharmacokinetic profiles of Rb1 following oral and intravenous administration

Plasma concentrations of Rb1 and Rd in CON, HFD and DM rats following oral dose of Rb1 were measured (Figure 2A and B) and main pharmacokinetic parameters were estimated (Table 1). The results showed that diabetes significantly enhanced the systemic exposure of Rb1, evidenced by significantly higher Cmax and AUC0-∞. A long half-life of
Rb1 was estimated in each group while DM rats showed a relatively shorter $T_{1/2}$. Collectively, oral bioavailability of Rb1 in DM rats (F%, Table 1) was significantly increased by 2.50 and 2.88 fold of CON and HFD rats respectively. It was also found that diabetes showed a trend to increase Rd concentration, significant increase was observed in 10 h following dose, but $C_{\text{max}}$ and $AUC_{0-\infty}$ of Rd were slightly altered by diabetes. In contrast to diabetic rats, feeding with high fat diet (HFD) decreased plasma exposure of Rb1 and Rd compared with CON rats, the significant decreases in $C_{\text{max}}$ of Rd were obtained.

Pharmacokinetic profiles of Rb1 in rats were also measured following intravenous administration of Rb1 (Figure 2C). Diabetes significantly decreased plasma concentration of Rb1 following intravenous dose, accompanied by significant decrease of $AUC_{0-\infty}$ value and an increase of systematic Rb1 clearance. Significant decrease in exposure of Rd was also observed in diabetic rats (Table 1, Figure 2D). All these results indicated that the increased exposure of Rb1 following oral dose of Rb1 in diabetic rats can not be attributed to alteration in systematic clearance.

To exclude hepatic contribution to the alteration of systemic exposure following oral administration, plasma concentration of Rb1 in portal vein was evaluated (Figure 2E). It was consistent with our expectation that portal Rb1 concentration in diabetic rats was higher than those in CON rats and HFD rats, leading to marked increases in $AUC_{0-8h}$.
of Rb1 (3.79±0.88 µg·h /ml in DM rats, 2.72±0.85 µg·h /ml in CON rats and 2.58±0.54 µg·h /ml in HFD rats), inferring that Rb1 absorption was enhanced under diabetic condition.

**Rb1 absorption via intestinal wall**

Accumulative Rb1 absorption was assessed using in situ intestinal perfusion (Figure 3A) and corresponding P_{eff} values (Figure 3B) were estimated. Results showed that accumulative Rb1 absorption was significantly enhanced by diabetes. Significant increases were observed in P_{eff} values of Rb1 at 45-min and 105-min following perfusion. These results inferred that diabetes enhanced Rb1 absorption via intestinal wall, which was in line with in vivo findings.

**Rb1 biliary and urinary excretion studies**

In most cases, ginsenosides were supposed to have a rapid and extensive biliary excretion (43-100% of intravenous dose), while Rb1 is relatively lower (approximately 10%) (Liu et al. 2009). However, in current study, the contribution of biliary excretion to the overall Rb1 elimination was very limited (less than 1% of intravenous dose), although it is of interest that accumulative biliary excretion of Rb1 and Rd in diabetic rats is significantly lower than CON rats (Figure 3C and D).

In contrast, approximately 87.8% of intravenously administrated
Rb1 were excreted via urine in diabetic rats, which is markedly higher than that of CON rats and HFD rats (50.7% and 43.9% respectively, Figure 3E). Significant increases in Rb1 excretion via urine may largely contribute to the low systemic exposure of Rb1 after intravenous administration under diabetic status. Urinary excretion of Rd via urine in DM rats showed a trend to decrease (Figure 3F), but no significance was observed.

**Metabolism of Rb1 by microsome and intestinal microflora**

To further elucidate whether metabolic enzymes contributed to the change of Rb1 pharmacokinetic profile in diabetic rats, Rb1 depletion via both hepatic and intestinal microsome were investigated. Inconsistent with our expectation, Rb1 metabolism by hepatic and intestinal microsome were extremely weak although DM group showed greater Rb1 depletion (Figure 4A and B), indicating that Rb1 metabolism by microsomes is not a dominant pathway of Rb1 elimination.

It was confirmed that deglycosylation of ginsenosides was the major biotransformation pathway after oral dosing. To gain more insights into the alterations of Rb1 biotransformation by diabetes, formation of Rb1 metabolites incubated with intestinal contents from CON, HFD and DM rats were identified.

When Rb1 incubated with the intestinal microflora of large intestine,
C-K was the main metabolite of Rb1 which was in accordance with previous report (Tawab et al. 2003). However, the generations of C-K were extremely reduced in DM rats (Figure. 4C), suggesting that deglycosylation of Rb1 was inhibited under diabetic condition.

Generation of Rd in reaction system varied with time process. Rd initially reached to a high level and then decreased steadily in CON and HFD group (Figure. 4D) indicating that Rd was produced rapidly and then further decomposed to other secondary metabolites, i.e., F2 and C-K. Similar profile was observed in the generation of F2 (Figure. 4E). By contrast, Rd content in DM group increased from a low level and then remained stable high without further decrease (Figure. 4D), indicating that formation of Rd under diabetic status was inhibited and secondary deglycosylation was abrogated (Figure. 4C and E). As a result, Rd accumulation during the incubation in DM group lead to higher AUC$_{0-12h}$ of Rd compared to CON group (Figure. 4F) and further lead to extremely low generation of C-K and F2 (Figure. 4C, E and F).

When incubated with the content of small intestine (Figure. 4G), Rb1 were mainly degraded to Rd by stripping one sugar moiety. Similarly, the generation of Rd in DM group was much lower than those in CON and HFD group. Based on these findings, it is demonstrated that considerable degradation of Rb1 in gastrointestinal tract of normal rats was drastically attenuated by diabetes, which partly remained in HFD...
Rb1 uptake and transport across cell monolayer

In order to identify whether increased Rb1 absorption was associated with transporters, effect of P-gp and Oatp inhibitors on Rb1 transport were investigated. Figure 5A showed Rb1 transport across monolayers of MDCK and P-gp over-expressed MDR1-MDCK cells. Rb1 efflux ratios were less than 2 and P-gp inhibitors (verapamil and cyclosporine A) did not affect the efflux ratio either indicating that Rb1 was not a substrate of P-gp. Data from Rb1 uptake showed that neither Oatp 1a4 inhibitors (BSP and rifampicin) nor Oatp 1a5 inhibitors (naringin and levofloxacin) exhibited appreciable impact on Rb1 uptake by Caco-2 cells (Figure 5B).

It is known that gut permeability is one of the determining factors for drug transport. Thus, the transport study of FD4 and FLU across Caco-2 were performed to evaluate the alteration of paracellular permeability under diabetic condition. Caco-2 monolayer was pre-treated with DMEM supplemented with 10% rat serum (CON, HFD and DM) for 8 days to simulate the internal environment. TEER of cell monolayer showed incubation with DM serum remarkably decreased TEER (DM, $508\pm14.1\ \Omega\cdot\text{cm}^2$ vs CON, $725\pm15.9\ \Omega\cdot\text{cm}^2$, P<0.01). Furthermore, incubation with 10% DM serum significantly increased transport of FD-4 and FLU across Caco-2 monolayer in the A to B direction (Figure 5C).
which indicated that paracellular permeability of the monolayer was increased under diabetic condition. Similarly, incubation with 10% DM serum significantly increased Rb1 transport across Caco-2 monolayer in the A-B and B-A direction (Figure 5D), the extent of increase in A-B direction was larger than that in B-A direction.

**Discussion**

Previous reports mainly focused on the anti-diabetic actions of ginsenosides (Park K et al 2002; Park M et al 2008; Shang et al. 2008) as well as their pharmacokinetic properties under normal condition. However, very little was known about pharmacokinetic behaviors of ginsenosides under diabetic condition. The main finding of the present study was that diabetes significantly increased Rb1 systemic exposures following oral administration. Although ginsenosides were considered to be of poor absorption, diabetes increased exposure level of Rb1 following oral administration which is beneficial to Rb1 treatment as an anti-diabetic remedy. Meanwhile, diabetes decreased systemic Rb1 exposures following intravenous administration and increased portal Rb1 concentration inferring that increased exposure of Rb1 following oral dose under diabetic condition at least partly was attributed to alterations in intestinal systems.
Data from *in situ* intestinal perfusion demonstrated that enhancement of Rb1 absorption was partly attributed to Rb1 transport across intestinal wall. It is reported that diabetes down-regualted intestinal P-gp function and Rh2 and C-K were identified to be the substrate of P-gp although that was controversial (Liu et al. 2009; Yang et al. 2011&2012), inferring that increased Rb1 absorption may be associated with down-regulation of intestinal P-gp under diabetic condition. Unfortunately, our data showed Rb1 transport across MDR1-MDCK monolayer was not regulated by P-gp. Additionally, Rb1 seemed not to be a substrate of Oatps. Several reports showed that some oxygenated metabolites of Rb1, Rg3 and Rh2 mediated by CYP450s had been identified (Qian et al. 2005a, 2005b&2006). Our results showed that its contribution to Rb1 depletion can be negligible due to the extreme poor capacity on Rb1 metabolism in hepatic and intestinal microsome. Similar results were found in PPT-type ginsenosides (Hao et al. 2010). Compared to some deglycosylated metabolites such as Rh1 and Rf, Re as well as Rb1 was not easy to be metabolized by CYP3A4, implying that efficiency of oxygenation was reduced due to sugar moities at C20 site. Taken together, Rb1 disposition may not be markedly regulated by transporters or CYP450s.

Intestinal microflora mediated deglycosylation is considered as the main metabolic pathway of ginsenosides (Qian et al. 2006). Under normal status, Rb1 was metabolized to Rd, which was further metabolized to F2
and C-K. Our results showed that further deglycosylation of Rd was drastically abrogated under diabetic condition, leading to the accumulation of Rb1 and Rd in intestinal lumen, which may directly contribute to the increased exposure of Rb1 and Rd (Figure 2A and B) and decreased exposure of F-2 and C-K (data not shown) in vivo. Deglycosylation of Rb1 in HFD rats was less inhibited compared to diabetic rats which reflected by higher F2 accumulation and C-K formation (Figure 4C, E and F). In addition, Rd may be the only detectable metabolite following intravenous administration of Rb1, inferring that deglycosylation of Rb1 also occurred in vivo. However, AUC_{0-72h} of Rd in CON rats only account for approximately 0.06% of i.v. dose while that of DM rats was much lower.

On one hand, the major factor limiting intestinal absorption of ginsenosides is poor membrane permeability which mainly due to high molecular weight and hydrogen bond counts (Liu et al. 2009). On the other hand, the increased paracellular permeability of intestinal epithelium under diabetic condition may benefit the Rb1 absorption. Caco-2 monolayer was pre-cultured with rat serum to simulate the intestinal environment. FLU and FD-4 are used as markers of trans-epithelial transport. The results demonstrated that cultured with diabetic serum significantly decreased TEER and increased FD-4, FLU and Rb1 transport across Caco-2 monolayer, inferring that enhancement
of Rb1 intestinal absorption by diabetes partly came from the impairment of intestinal integrity. Occludin and ZO-1 are known as important tight conjunction proteins in integrity function. It is still need to be explored if Rb1 absorption is associated with the distribution of occludin and ZO-1 (Cani et al. 2008).

The present study also showed that urinary excretion of Rb1 (approximately 87.8% of dose) in diabetic rats was significantly higher that those of CON rats (50.7%) and HFD rats (43.9%), inferring that the increased urinary excretion of Rb1 may result in the lower plasma exposure of Rb1 following intravenous Rb1 administration. For the higher renal clearance of Rb1, increased urine volume is supposed to be an important factor. Hyper-filtration is the marked feature of diabetic nephropathy, which also become a reason leading to high renal clearance of Rb1. Apart from this, although Rb1 was proved not to be a substrate of P-gp and Oatp, other transporters may have the potential to mediate Rb1 transport, for instance, SGLT1 (Xiong et al. 2009). Therefore, further study should investigate the effect of diabetic nephropathy on Rb1 elimination and the possible regulator SGLT1/2 in renal tubule.

Apart from normal control, HFD rats were set as the other control group due to the metabolic syndrome induced by high-fat diet such as hyperlipidemia as well as insulin resistance (Liu et al. 2014). High-fat diet treatment exhibited a trend to decrease the oral exposure of Rb1 and
Rd compared to CON rats, which was opposite to diabetic rats (Figure 2A and B, Table 1). Similar AUC$_{0-\infty}$ and clearance as CON rats were showed in HFD rats following Rb1 intravenous administration (Table 1). Portal concentration and intestinal absorption of Rb1 was mildly lower than CON rats (Figure 2E and 3A), which may partly interpret the decreased oral Rb1 exposure. In vitro, HFD serum treatment showed a trend to increase the permeability of Caco-2 monolayer but failed to increase Rb1 transport (Figure 5C and D). This indicated that paracellular pathway did not play a key role in the alteration of Rb1 exposure in HFD rats. Further investigations are required.

In conclusion, diabetes significantly increased plasma exposure of Rb1 following oral administration. Combined effect of increased Rb1 intestinal absorption and inhibited Rb1 deglycosylation may play an important role. The current study provided an important reference for clinical use of ginseng.
Authorship Contributions

Participated in research design: C. Liu, L. Liu, and X. Liu.

Conducted experiments: C. Liu, M. Hu, M. Zhang, J. Zhang, F. Li, Y. Li, and P. Xu

Performed data analysis: C. Liu, Z. Zhong, H. Guo, Y. Chen, L. Liu and J. Li

Wrote or contributed to the writing of the manuscript: C. Liu, and X. Liu.
Reference


**Footnotes**

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Legends for Figures

Figure 1. Structures of 20-(S)-protopanaxadiol (PPD-type) (A) and the proposed biotransformation pathway of Rb1 induced by intestinal microflora (B). C-K, Compound-K; Glc, β-D-glucose.

Figure 2. Plasma concentrations of Rb1 and its main metabolic product Rd in CON, HFD, and DM rats after Rb1 administration orally (A, B; Rb1 100 mg/kg) and intravenously (C, D; Rb1 10 mg/kg); E, Rb1 concentration in portal plasma following oral dose (100 mg/kg). Values are expressed as mean±S.E.M. (n = 5), #P<0.05, ##P<0.01 vs. CON.

Figure 3. Accumulative absorption of Rb1 (A) and corresponding P eff values (B) were measured using in situ, single-pass perfusion of the jejunum in experimental rats; biliary (C, D) and urinary (E, F) excretion of Rb1 and Rd after intravenous Rb1 administration (10 mg/kg). Values are expressed as mean±S.E.M. (n = 5), #P<0.05, ##P<0.01 vs. CON.

Figure 4. Metabolism of Rb1 in hepatic (A) and intestinal (B) microsomes from CON, HFD and DM rats in vitro. Depletion of Rb1 (200 nM) in hepatic microsome was measured with incubation times of 0, 30, 60, and 120 min while depletion of Rb1 (50 nM) in intestinal
microsome was measured with incubation times of 0, 60, and 120 min. Concentration-time profiles of C-K (C), Rd (D) and F2 (E) and corresponding AUC$_{0-12h}$ value (F) metabolized by large intestinal microflora from CON, HFD, and DM rats. G, concentration-time profile of Rd metabolized by content of small intestine. Values are expressed as mean±S.E.M. (n = 4), #P<0.05, ##P<0.01 vs. CON.

Figure 5. A, P$_{app}$ value of Rb1 (2 μM and 10 μM) transport across monolayers of MDCK and MDR1-MDCK cells with and without P-gp inhibitors (Verapamil 50 μM or Cyclosporine A 20 μM), efflux ratios were shown over the bars; B, cellular uptake of Rb1 at 50 μM by Caco-2 cells with and without Oatp inhibitors namely BSP (bromosulfophthalein, 100 μM), RFP (Rifampicin, 20 μM), NRG (Naringin, 1 mM) and LVFX (Levofloxacin, 500 μM) for 5 min; C, transport of FD4 (1 mg/mL) and FLU (0.1 mg/mL) from apical to basal (A→B) direction were measured to evaluate the permeability of Caco-2 monolayer cultured with 10% rat serum; D, Rb1 (10 μM) transport across the Caco-2 monolayer cultured with 10% rat serum from both A→B and B→A direction. Caco-2 cells were pre-treated with 10% rat serum for 8 days. Values are expressed as mean±S.E.M. (n = 3-5). #P<0.05, ##P<0.01 vs. CON.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>CON</th>
<th>HFD</th>
<th>DM</th>
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</thead>
<tbody>
<tr>
<td><strong>Rb1 100 mg/kg p.o.</strong></td>
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<tr>
<td>AUC(_{0-\infty}) (µg/ml•h)</td>
<td>42.47±7.26</td>
<td>35.61±5.24</td>
<td>75.63±12.10*</td>
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<td>C(_{\text{max}}) (µg/mL)</td>
<td>1.73±0.25</td>
<td>1.20±0.28</td>
<td>3.09±0.34*</td>
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<tr>
<td>T(_{\text{max}}) (h)</td>
<td>8.80±0.80</td>
<td>8.00</td>
<td>8.40±0.40</td>
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<tr>
<td>T(_{1/2}) (h)</td>
<td>24.00±1.10</td>
<td>25.03±1.66</td>
<td>20.08±1.42</td>
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<tr>
<td>MRT (h)</td>
<td>25.03±0.54</td>
<td>27.01±1.09</td>
<td>24.46±0.94</td>
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<td>F (%)</td>
<td>0.90</td>
<td>0.78</td>
<td>2.25</td>
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<tr>
<td>CL (L/h/kg)</td>
<td>2.38±0.49</td>
<td>2.56±0.40</td>
<td>1.37±0.28</td>
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<tr>
<td><strong>Rd</strong></td>
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<tr>
<td>AUC(_{0-\infty}) (µg/ml•h)</td>
<td>7.59±2.25</td>
<td>2.40±0.53</td>
<td>6.85±0.88</td>
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<td>C(_{\text{max}}) (µg/mL)</td>
<td>0.45±0.08</td>
<td>0.21±0.05*</td>
<td>0.65±0.07</td>
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<td>T(_{\text{max}}) (h)</td>
<td>8.80±0.80</td>
<td>8.00</td>
<td>8.80±0.49</td>
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<tr>
<td>T(_{1/2}) (h)</td>
<td>16.78±3.24</td>
<td>12.34±3.21</td>
<td>9.27±1.91</td>
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<tr>
<td>MRT (h)</td>
<td>22.31±2.71</td>
<td>18.18±1.53</td>
<td>15.53±1.71</td>
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<tr>
<td><strong>Rb1 10 mg/kg i.v.</strong></td>
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<tr>
<td>AUC(_{0-\infty}) (µg/ml•h)</td>
<td>472.3±15.2</td>
<td>458.1±22.0</td>
<td>335.6±22.1**</td>
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<td>V(_d) (ml/kg)</td>
<td>520.5±15.9</td>
<td>523.9±20.0</td>
<td>479.2±17.5</td>
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<td>T(_{1/2}) (h)</td>
<td>18.19±0.55</td>
<td>17.64±1.00</td>
<td>11.26±0.70**</td>
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<td>MRT (h)</td>
<td>19.42±0.32</td>
<td>19.50±0.61</td>
<td>15.80±0.93**</td>
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<td>CL (mL/h/kg)</td>
<td>19.88±0.67</td>
<td>20.80±1.31</td>
<td>29.86±2.26**</td>
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<tr>
<td><strong>Rd</strong></td>
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<tr>
<td>AUC(_{0-72h}) (µg/ml•h)</td>
<td>20.05±6.14</td>
<td>10.67±1.19</td>
<td>5.08±0.35*</td>
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</table>

AUC\(_{0-\infty}\), area under time-concentration curve; C\(_{\text{max}}\), maximum concentration; T\(_{\text{max}}\), time to C\(_{\text{max}}\).
$T_{1/2}$, half life; MRT, mean residence time; F, bioavailability; CL, clearance; Vd, volume of distribution. Values are expressed as mean±S.E.M. (n = 4-5), *P<0.05, **P<0.01 vs. CON.
**Figure 2**

(A) Concentration of Rb1 in plasma over time for different groups: CON, HFD, and DM. The graphs show a significant increase in Rb1 concentration over time, with the highest levels observed in the HFD group.

(B) Concentration of Rd in plasma over time for different groups: CON, HFD, and DM. The graphs show a decrease in Rd concentration over time, with the HFD group having the lowest levels.

(C) Concentration of Rb1 in plasma over time for different groups: CON, HFD, and DM. The graphs show a decrease in Rb1 concentration over time, with the DM group having the lowest levels.

(D) Concentration of Rd in plasma over time for different groups: CON, HFD, and DM. The graphs show an increase in Rd concentration over time, with the HFD group having the highest levels.

(E) Concentration of Rb1 in plasma over time for different groups: CON, HFD, and DM. The graphs show a fluctuation in Rb1 concentration, with the HFD group having the highest levels at certain time points.
Figure 3

- A: Accumulative absorption (μg/cm²) over time (min) for CON, HFD, and DM groups.
- B: Peff (×10³ cm/min) at 45 min and 105 min for CON, HFD, and DM groups.
- C: Accumulative biliary excretion of Rb1 (μg) over time (h).
- D: Accumulative biliary excretion of Rd (μg) over time (h).
- E: Accumulative urinary excretion of Rb1 (μg) over time (h).
- F: Accumulative urinary excretion of Rd (μg) over time (h).
Figure 4