Intestinal Absorption and Metabolism of Epimedium Flavonoids in Osteoporosis Rats

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Abbreviations: BCRP, breast cancer resistance protein; CBG, cytosolic β-glucosidase; MRP, multidrug resistance-associated protein; LPH, lactase phlorizin hydrolase; P\textsuperscript{app}, apparent permeability coefficient; P-gp, P-glycoprotein; RPI, rearrangement product of icariin; EP, eliminative percentage.
Abstract

Herbal *Epimedium* is a traditional Chinese medicine used to treat osteoporosis. Its main pharmacological ingredients are flavonoids. In previous studies conducted in healthy animals, we showed that epimedium flavonoids could be hydrolyzed into secondary glycosides or aglycon by intestinal flora or enzymes, thereby enhancing their absorption and anti-osteoporosis activity. To study the medicine in the pathological state, epimedium flavonoids were incubated with intestinal mucosa and feces *in vitro* and intestinal perfusion *in situ* to explore the differences in absorption and metabolism between sham and osteoporosis rats. For osteoporosis rats, the hydrolysis rates of icariin, epimedin A, epimedin B, and epimedin C incubated with intestinal flora for 1 h reduced by 0.19, 0.26, 0.19, and 0.14, respectively, compared to that in sham rats. The hydrolysis rates of icariin, epimedin A, epimedin B, and epimedin C incubated with duodenal enzymes for 1 h reduced by 0.21, 0.24, 0.08, and 0.31, and with jejunal enzymes by 0.13, 0.09, 0.07, and 0.47, respectively, compared to the sham group. Additionally, the apparent permeability coefficient and elimination percentage of the four epimedium flavonoids in the duodenum, jejunum, ileum, and colon decreased by 29%–44%, 32%–50%, 40%–56%, and 27%–53% compared to that in sham rats, respectively. The main metabolites of the four epimedium flavonoids were the same for the two groups after intestinal perfusion, or flora and enzyme incubation. In conclusion, the amount and activity of intestinal flora and enzyme changed in ovariectomized rats, which affected the intestinal absorption and hydrolysis of epimedium flavonoids whose structures contain 7-glucose.
Introduction

Osteoporosis is characterized by the reduction and deterioration of the bone microarchitecture, leading to increased bone frailty and susceptibility to fracture. The worldwide incidence of osteoporosis is increasing, and can be an economic burden on both families and societies (Kanis et al., 1994; Johnell and Kanis, 2006). Currently, 200 million people worldwide have osteoporosis. A large number of people also have low bone mass, placing them at an increased risk for developing osteoporosis. As the population ages, these numbers will increase. A majority of those with osteoporosis are women. Of people older than 50 years, one in two women and one in eight men are predicted to have an osteoporosis-related fracture in their lifetime. Drugs being developed or used for treating osteoporosis include estrogen replacement therapy, calcitonin, selective estrogen receptor modulators, and diphosphate. Although these drugs prevent bone resorption, their effects on bone formation are extremely small (Abrahamsen et al., 2014; Bandeira et al., 2014; Chen and Kubo, 2014; Kulak et al., 2014).

Herbal *Epimedium*, a popular traditional Chinese medicine, has been used to treat osteoporosis in East Asian countries for over 2000 years (Nelson et al., 2002; Wu et al., 2003). In the perspective of modern medicine, the main pharmacological ingredients of Herbal *Epimedium* were found to be various prenylated epimedium flavonoids (including icarin, epimedin A, epimedin B, epimedin C, and baohuoside I) (Figure 1). Modern research has also confirmed that prenylated epimedium flavonoids can regulate the balance between osteogenic and adipogenic differentiation of bone marrow stromal cells in ovariectomized rats by down-regulating the expression of DKK1 protein, thereby enhancing
bone formation, and can also prevent ovariectomy-induced bone loss (Xu et al., 2011; Zhang et al., 2007). However, the oral bioavailability of epimedium flavonoids is very low (Chen et al., 2014; Zhou et al., 2014; Wei et al., 2012).

Our previous study found that there are several reasons for this low bioavailability. Firstly, epimedium flavonoids have low absorptive permeabilities due to their physicochemical characteristics (Chen et al., 2008). Secondly, they are subject to efflux by the drug transporters in intestinal mucosa, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated protein (MRP) (Chen et al., 2014; Wolfgang et al., 2005). Thirdly, these flavonoids can be hydrolyzed by hydrolase in the intestinal mucosa and flora. Lactase phlorizin hydrolase (LPH) and β-glucosidase were confirmed as the main hydrolases in intestinal mucosa and flora, which could hydrolyze the epimedium flavonoids to secondary glycosides or aglycon (Cui et al., 2013; Qian et al., 2012; Zhao et al., 2010). The above results were obtained with healthy animals; however, the absorption and hydrolysis behavior in osteoporotic animal remains unknown.

Therefore, we have studied the absorption and hydrolysis of epimedium flavonoids under osteoporotic conditions. In this study, we explored the absorption and hydrolysis characteristics of icariin, epimedin A, epimedin B, epimedin C, and baohuoside I in osteoporosis rats. To analyze the hydrolysis rates of epimedium flavonoids and to detect their metabolites, they were incubated in vitro with rat intestinal mucosa and feces at 37°C. The absorption of epimedium flavonoids was investigated using an in situ intestinal perfusion model. With these methods, the absorption and hydrolysis characteristics of epimedium flavonoids in osteoporosis rats have been revealed, and the differences of
absorption and hydrolysis characteristics of epimedium flavonoids between sham and osteoporosis rats are discussed.
Materials and Methods

Ethics Statement

Animal welfare and experimental procedures were strictly in accordance with the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996) and the related ethics regulations of Jiangsu Provincial Academy of Chinese Medicine.

Reagents and chemicals

Icariin (purity > 98%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Epimedin A, epimedin B, epimedin C, and baohuoside I (all purities > 98%) were provided by the Laboratory of Pharmaceutical Preparation (Jiangsu Provincial Academy of Chinese Medicine, Nanjing, China). LPH antibody and β-actin were purchased from Acris Antibodies, Inc. (San Diego, CA, USA). ECL kit was obtained from KeyGen BioTECH (Nanjing, China). Testosterone (purity > 98%), K₂HPO₄, NaCl, (NH₄)₂SO₄, CaCl₂, MgSO₄·H₂O, Na₂CO₃, HCl, L-cysteine, L-ascorbic acid, eurythrol, tryptone and nutrient agar were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other materials (typically analytical grade or better) were used as received.

Animals

Eight-week-old female Sprague-Dawley rats with body weight of 170 - 250 g were obtained from the SLEK Lab Animal Center of Shanghai (Shanghai, China) and were housed under standard conditions of temperature, humidity, and light. Food and water were provided ad libitum. The rats were fasted overnight before the day of the experiment. Rats were randomly assigned to two groups: sham group (sham), ovariectomized group (osteoporosis).
After one week of acclimatization, rats were anesthetized with intraperitoneal (i.p.) injection of 300 mg chloral hydrate (Sinopharm®, China)/kg body weight and then both ovaries were removed. Rats were left untreated for 3 months to allow them to recover and develop osteopenia according to a previously published method (Wang et al., 2013). The sham rats underwent bilateral laparotomy but the ovaries were left in place. The result of bone mineral density (BMD) of femur in rats confirmed the osteoporosis model was available for subsequent experiments (Supplemental Table 1).

**Intestinal flora hydrolyze epimedium flavonoids**

Anaerobic culture medium were prepared as follows: K$_2$HPO$_4$ (37.5 mL, 0.78%), solution A (37.5 mL, 0.47% KH$_2$PO$_4$, 1.18% NaCl, 1.2% (NH$_4$)$_2$SO$_4$, 0.12% CaCl$_2$, 0.25% MgSO$_4$·H$_2$O), Na$_2$CO$_3$ (50 mL, 8%), L-cysteine (0.5 g), L-ascorbic acid (2 mL, 25%), eurythrol (1 g), tryptone (1 g) and nutrient agar (1 g) were mixed together and diluted with distilled water to 1 L. Then the solution was adjusted pH to 7.5 - 8.0 with 2 M HCl (Akao et al., 1996).

Fresh feces collected from SD rats were immediately homogenized in normal saline solution at the ratio of 1 g to 4 mL. The homogenate was filtered, and the filtrate of fresh feces (10 mL) was added anaerobic culture medium (90 mL) to obtain an intestinal flora cultural solution. Icariin, epimedin A, epimedin B, epimedin C or baohuoside I (1 mL; 2 mM) were then added to the intestinal flora cultural solution (9 mL), respectively.

After incubation for 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12 and 24 h at 37°C, the incubations were deproteinized by adding a 3-fold volume of acetonitrile. The samples were vortexed for 1 min and centrifuged for 15 min at 15,000 rpm. For HPLC-UV analysis, 400
μL aliquots of the supernatants were mixed with 100 μM internal standard testosterone (100 μL) which dissolved in acetonitrile. For UPLC/Q-TOF-MS analysis, the supernatants (150 μL) were purified using a C18 solid phase extraction cartridge (Agilent, Santa Clara, CA, USA). The cartridge was activated by methanol (3 mL), and was then balanced using water (2 mL). Intestinal perfusion samples (0.6 mL) were mixed with methanol (150 μL). The mixtures were centrifuged for 15 min at 13,000 rpm. Supernatants (0.6 mL) were added into the cartridge, and then the cartridge was eluted with water (5 mL) and methanol (5 mL). The elutropic methanol was collected and evaporated to dryness under a gentle stream of nitrogen at 30°C before being reconstituted in acetonitrile (450 μL).

The experiments were divided into the sham group and the osteoporosis group, fresh feces was collected from both groups at 3 months post-operation.

**Intestinal enzyme hydrolyze epimedium flavonoids**

After overnight food deprivation, rats were anesthetized by intramuscular injection of urethane (0.5 g/mL). An incision was made into the abdominal cavity to take out the small intestine, and the intestine was immediately preserved in cold saline. After being divided into duodenum, jejunum, ileum, and colon, the contents of the four segments were removed by flushing gently with saline (0°C). Intestinal mucosa was blunt scratched. Intestinal mucosa in different segments was immediately homogenized in normal saline solution at the ratio of 1 g to 4 mL. Homogenates of intestinal mucosa (10 mL) were mixed with cold saline (90 mL) to prepare the intestinal enzyme cultural solution (Aloys et al., 2003).

The next procedures and animal group assignment were the same as the experiments of intestinal flora hydrolyzing epimedium flavonoids. At the same time, protein was extracted
from frozen intestines by RIP A buffer containing 1 mM PMSF on the purpose of examining
the LPH expression.

**In situ intestinal perfusion assay of epimedium flavonoids**

The rats were fasted overnight but were provided with deionized water. After overnight
fasting, rats were anesthetized. The small intestine was exposed by midline incision; the
intestinal lumen was then gently flushed to remove intestinal contents and each of the four
segments (duodenum, upper jejunum, terminal ileum, and colon) of the intestine was
cannulated with two cannulas. The outlet of each segment was secured by ligation with silk
suture. The intestine was carefully arranged and continuously monitored to avoid kinks, and
a consistent flow should be ensured after cannulation. Saline-soaked cotton was used to
cover opened body cavities to prevent loss of fluids (Andlauer et al., 2000; Andlauer et al.,
2000; Andlauer et al., 2000; Chen et al., 2003; Liu et al., 2002; Higaki et al., 2004 ).

At 3-month ovariectomized postoperation, both sham rats and osteoporosis rats were
operated as above steps. To keep the temperature of the perfusate constant, the inlet cannula
was insulated and kept warm by a 37°C circulating water bath. A flow rate of 0.2 mL/min
was used, and the perfusate samples were collected every 30 min. The outlet concentration
of drug in the perfusate was determined by UPLC-UV, and the hydrolyzed products were
detected by UPLC/Q-TOF-MS. The preparation method of perfusate samples was the same
as the intestinal flora and enzyme samples. The initial concentration of the perfusate was 20
μM.

**Analytical Methods**

UPLC-UV is a rapid and efficient method for determining the contents of different samples.
However, impurities in the intestinal flora and enzyme samples would block a UPLC-UV system. On the other hand, the perfusate samples were cleaner than intestinal flora and enzyme samples. Therefore, HPLC-UV was used for determining the contents of epimedium flavonoids in intestinal flora and enzyme samples, while UPLC-UV was used for determining the contents of epimedium flavonoids in perfusate samples, and all the metabolites were identified by UPLC/Q-TOF-MS method.

UPLC-UV method: Waters Acquity UPLC system with photodiode array detector and Empower software; column, Acquity UPLC BEH C18, 1.7 μm, 2.1 × 50 mm (Waters, Milford, MA, USA); mobile phase A, acetonitrile; mobile phase B, water; gradient, 0 to 0.5 min, 25% A, 0.5 to 1.5 min, 25% to 28% A, 1.5 to 2.5 min, 28% A to 32%, 2.5 to 3.5 min, 32% A to 35% A, 3.5 to 4.5 min, 35% A to 55%, 4.5 to 5.5 min, 55% A to 95%, 5.5 to 6.0 min, 95% A; flow rate, 0.4 mL/min; column temperature, 30°C; injection volume, 5 μL; wavelength, 270 nm for epimedium flavonoids and 254 nm for internal standard testosterone. In general, these methods were selective and reproducible with day-to-day variability less than 2%. The accuracy and precision were greater than 92%. The tested linear response ranges for samples were 2.5 to 40 μM. Epimedium flavonoids exhibited good linearity within the selected concentration ranges with correlation coefficients (R²) between 0.9994 and 0.9999.

HPLC-UV method: System, Agilent 1260 HPLC-UV with photodiode array detector; column, ZORBAX SB-C18, 5 μm, 4.6 × 250 mm (Agilent, Santa Clara, CA, USA); mobile phase A, water; mobile phase B, acetonitrile; gradient, 0 to 6.5 min: 70% A, 6.5 to 10 min: 45% A, 10 to 14 min: 45% A, 14 to 15 min: 70% A; flow rate, 1 mL/min; wavelength, 270
nm for epimedium flavonoids and 254 nm for internal standard testosterone; injection volume, 10 μL. In general, these methods were selective and reproducible with day-to-day variability less than 2%. The accuracy and precision were greater than 97%. The tested linear response ranges for all flavonoids were 6.25 to 100 μM, respectively. Epimedium flavonoids exhibited good linearity within the selected concentration ranges with $R^2$ between 0.9992 and 0.9997.

UPLC/Q-TOF-MS method: Waters Synapt G2-S Q-TOP mass spectrometer (Waters Corp., Milford, MA, USA) equipped with electrospray ionization (ESI) source. The samples were separated on a 1.7 μm, 2.1 × 50 mm Acquity UPLC BEH C18 column (Waters, Milford, MA, USA). The mobile phase consisted of acetonitrile (A) and water (B). The gradient program of epimedium flavonoids was as follows: 0 to 1 min, 5% A, 1 to 1.5 min, 5% to 25% A, 1.5 to 3 min, 25% A, 3 to 4 min, 25% to 28% A, 4 to 5 min, 28% to 32% A, 5 to 6 min, 32% to 35% A, 6 to 7 min, 35% to 55% A, 7 to 8 min, 55% to 95% A, 8 to 9.5 min, 95% A, 9.5 to 10 min, 95% to 5% A, the flow rate was 0.4 mL/min; the column temperature was maintained at 30°C; the injection volume was 2 μL. The tested linear response ranges for samples were 1.25 to 40 μM. Epimedium flavonoids exhibited good linearity within the selected concentration ranges with $R^2$ between 0.9992 and 0.9996.

For MS analysis, the ESI source was operated in the positive ion mode. The capillary and cone voltages were 3,000 and 20 V, respectively. The tapered bore gas was 50 L/h, the desolvation gas (nitrogen) was set to 600 L/h at 400°C, and the source temperature was 100°C. Mass range was scanned from 100 to 1200 Da. Multireaction monitoring mode (MRM); low energy: 6V, high energy: 20-30 V.
The monitored ion pairs of metabolites of the four flavonoids are shown in Supplemental Table 2. The metabolites of icariin were M1 and M2. M1 showed the presence of [M+H]$^+$ at \(m/z\) 515.18, and its main fragment ion peaks contained \(m/z\) 313.07 and 369.13. M2 showed the presence of [M+H]$^+$ at \(m/z\) 677.24, and its fragment ion peaks were predominantly \(m/z\) 313.07 and 369.13. The metabolite of epimedin A showed the presence of [M+H]$^+$ at \(m/z\) 677.24, and its main fragment ion peaks contained \(m/z\) 369.13 and 313.07. The metabolite of epimedin B contained [M+H]$^+$ at \(m/z\) 647.23, and its fragment ion peaks mainly included \(m/z\) 369.13 and 313.07. The metabolite of epimedin C showed the presence of [M+H]$^+$ at \(m/z\) 661.25, and its fragment ion peaks mainly included \(m/z\) 515.19, 369.13 and 313.07.

**Data Analysis**

For analyzing the hydrolysis behavior of epimedium flavonoids in intestinal flora and enzyme incubation, the hydrolysis rate of epimedium flavonoids was calculated using the following equation:

\[
Hydrolysis rate = \frac{A_t - A_r}{A_t} \quad [1]
\]

In the equation [1], \(A_t\) is the total amount of epimedium flavonoids before hydrolysis; \(A_r\) is the residual amount of epimedium flavonoids after hydrolysis.

For analyzing the absorption behavior of epimedium flavonoids in intestinal perfusion, the absorption of drug was measured by the rate of its disappearance, and the apparent permeability coefficient (\(P^*_{app}\)) was determined through the rate of drug disappearance. The \(P^*_{app}\) was calculated using the following equation:

\[
P^*_{app} = -v \cdot \frac{1}{2\pi r l} \ln \left( \frac{\rho_{out} \cdot V_{out}}{\rho_{in} \cdot V_{in}} \right) \quad [2]
\]
In the equation [2], $V_{in}$ and $V_{out}$ (mL) are volumes of poured and collected testing solution, respectively; $v$ is the perfusion velocity; $\rho_{out}$ and $\rho_{in}$ (g/L) are concentrations of inlet and outlet solutions, respectively; $l$ (cm) is the length of the perfused bowel (typical length is 10 cm), $r$ (cm) is the cross-sectional radius of the perfused bowel. Values were indicated as mean ± S.D.

**Statistical Analysis**

Student's $t$-test (Microsoft Excel) was used to analyze the data. The prior level significance was set at $P < 0.05$. 
Results

Hydrolysis of epimedium flavonoids by intestinal flora

When incubated with intestinal flora of sham and osteoporosis rats, icariin, epimedin A, epimedin B, and epimedin C were hydrolyzed rapidly. HPLC-UV demonstrated that each of them had only one metabolite (Supplemental Figure 2). UPLC/Q-TOF-MS revealed that the metabolites of the four flavonoids were the 7-deglucosed products, baohuoside I (icariin), sagittatoside A (epimedin A), sagittatoside B (epimedin B), and 2"-O-rahmonosylicarside II (epimedin C). The metabolites of the sham and osteoporosis groups were the same.

Although the metabolites in the two groups were similar, the hydrolysis rates were different. To measure the hydrolysis rate of epimedium flavonoids, rates measured at logarithmically varying concentrations of flavonoids (Y) and time (X/h) were fit to regression equations. Larger slopes indicate a more rapid hydrolysis. The hydrolysis velocities of icariin, epimedin A, epimedin B, and epimedin C (slope values = 0.297, 0.264, 0.279, and 0.164, respectively) in sham rats were greater than in osteoporosis rats (slope values = 0.237, 0.212, 0.217, and 0.090, respectively) (Table 1). The hydrolysis of icariin was the most rapid of the four epimedium flavonoids in both groups, indicating that the 7-glucose in icariin, which has two glycosylations, was more easily hydrolyzed by intestinal flora than that in epimedin A, epimedin B, and epimedin C, all of which have three glycosylations.

The extent of hydrolysis of epimedium flavonoids incubated with intestinal flora also varied between sham and osteoporosis rats. After being incubated for 1 h with intestinal flora, 0.65, 0.63, 0.59, and 0.51 of icariin, epimedin A, epimedin B, and epimedin C, respectively, was metabolized in sham rats. In osteoporosis rats the extent of hydrolysis of icariin,
Icariin, epimedin A, and epimedin B were 0.56, 0.37, 0.40, and 0.37, respectively. Icariin, epimedin A, and epimedin B could not be detected after 4 h of incubation in intestinal flora from sham rats, whereas, they still could be detected in the samples of intestinal flora from osteoporosis rats after 6 h. The hydrolysis of epimedin C was slower than the other three compounds and it could still be detected after 8 h of incubation with intestinal flora from both sham and osteoporosis rats. Nevertheless, the extent of hydrolysis of epimedin C incubated with intestinal flora of sham rats was 22.03% higher than that of osteoporosis group. Baohuoside I was not hydrolyzed by the intestinal flora of either sham rats or osteoporosis rats (Figure 2). Overall, the intestinal flora of the osteoporosis group exhibited slower hydrolysis than did the sham group.

Hydrolysis of epimedium flavonoids by the intestinal enzymes from four different intestinal segments

Epimedium flavonoids were mainly hydrolyzed by duodenum and jejunum enzymes in the sham and osteoporosis groups. The metabolites were identified by UPLC/Q-TOF-MS. The metabolites in the two groups were the same. Furthermore, after intestinal enzyme incubation, the metabolites of epimedin A, epimedin B, and epimedin C were the same as that after intestinal flora incubation. However, icariin had an additional metabolite after intestinal enzyme incubation (Supplemental Figure 2). This metabolite has the same m/z of protonated molecular ions and daughter ions, suggesting that this metabolite of icariin might be a rearrangement product of icariin (RPI). The results indicate that hydrolase might be different in intestinal fecal and mucosa.

The hydrolysis rates of epimedium flavonoids incubated with four different intestinal
segments from sham and osteoporosis rats were calculated. As shown in Figure 3 and Table 2, the hydrolysis rates of epimedium flavonoids incubated with intestinal segments from osteoporosis rats were lower than those from sham rats. After 1 h incubation with duodenum, jejunum, ileum, or colon enzymes, the hydrolysis rates of icariin for the sham group were 0.89, 0.85, 0.040, and 0.0050, respectively. The hydrolysis rates of icariin for the osteoporosis group were 0.68, 0.72, 0.0030, and 0, respectively. With duodenum and jejunum enzyme incubation, the hydrolysis of icariin by the sham group was completed at 1.5 h, while the hydrolysis was not complete until 2 h in the osteoporosis group. The hydrolysis rates of epimedin A, after 1 h incubation with duodenum, jejunum, ileum, and colon enzymes were 0.39, 0.33, 0.040, and 0.020, respectively, for the sham group and 0.15, 0.24, 0, and 0, respectively, for the osteoporosis group. For epimedin B, the rates for the sham group were 0.30, 0.16, 0.21, and 0, respectively, and for the osteoporosis group were 0.22, 0.090, 0, and 0, respectively. For epimedin C, the rates of the sham group were 0.63, 0.70, 0.090, and 0, respectively, and of the osteoporosis group were 0.32, 0.23, 0.070, and 0, respectively.

The metabolites that were generated were also measured to evaluate the hydrolysis capability of intestinal enzymes. As shown in Figure 4, the amounts of metabolites of epimedium flavonoids incubated with four different intestinal segments were higher in the sham rats than the osteoporosis rats. Metabolites of icariin, baohuoside I and RPI, were detected after incubation with enzyme from duodenum, jejunum, and ileum for both groups. After incubation with colon enzymes, only baohuoside I could be found in the sham group, and RPI was not detected in either group. Moreover, the amount of baohuoside I increased...
with time; however, the amount of RPI increased, then declined, and finally disappeared, indicating RPI might be transferred to baohuoside I over time. Epimedin A, epimedin B, and epimedin C have similar structures, suggesting their hydrolysis mechanisms might be similar. Their metabolites, sagittatoside A, sagittatoside B, and $2''$-$O$-rahmonosylicarside II, respectively, were observed upon incubation with duodenum, jejunum, and ileum enzymes in the sham group, but they could only be detected upon incubation with duodenum and ileum enzymes in the osteoporosis group. No hydrolysis of baohuoside I was observed in any intestinal segment.

**Absorption and metabolism of epimedium flavonoids in an *in situ* intestinal perfusion model**

The above studies focused on the *in vitro* hydrolysis of epimedium flavonoids. In this section, we used an *in situ* intestinal perfusion model to study whether osteoporosis affected the *in situ* intestinal absorption and hydrolysis of epimedium flavonoids. The epimedium flavonoids could be absorbed and metabolized in two ways, either directly as the unchanged molecule or following hydrolysis by intestinal enzymes, such as LPH, and then absorbed as metabolites. Apparent permeability coefficient ($P_{app}^*$) and eliminative percentage ($10 \text{ cm}\% \text{ EP}$; 10 cm intestinal segments) were used to characterize the absorption and hydrolysis of epimedium flavonoids for sham and osteoporosis rats.

The metabolites in perfusates for the two groups were the same and were consistent with the metabolites observed after incubations with intestinal enzyme (Supplemental Figure 2), further confirming that the hydrolases might be different in intestinal feces and mucosa.
The results of absorption and hydrolysis of epimedium flavonoids in the in situ intestinal perfusion model are shown in Figures 5 ~ 8. As shown in Figure 5, in both groups, the $P^*_{app}$ and 10 cm% EP values of icariin became progressively less from duodenum to colon. In the sham group, the $P^*_{app}$ values of icariin in duodenum, jejunum, ileum, and colon were $5.59 \pm 0.57$, $5.26 \pm 0.74$, $1.30 \pm 0.18$, and $0.50 \pm 0.09$, respectively; and the 10 cm% EP values of icariin were $72.26 \pm 7.89\%$, $69.09 \pm 8.29\%$, $19.66 \pm 2.93\%$, and $9.48 \pm 1.50\%$, respectively. For the osteoporosis group, the $P^*_{app}$ values of icariin in duodenum and jejunum, and the 10 cm% EP values of icariin in the four intestinal segments were significantly lower than those of sham group (vs. the corresponding bowel, $P < 0.05$). The $P^*_{app}$ values of icariin in four intestinal segments were $3.87 \pm 0.46$ (duodenum), $3.57 \pm 0.55$ (jejunum), $0.73 \pm 0.60$ (ileum), $0.36 \pm 0.29$ (colon), and the 10 cm% EP values were $50.20 \pm 5.34\%$, $44.17 \pm 6.18\%$, $9.01 \pm 1.35\%$, and $4.72 \pm 0.76\%$, respectively. At the same time, the amounts of metabolites were significantly different. The metabolites baohuoside I and RPI were found in all four intestinal segments of the sham group. However, although baohuoside I could be detected in all the four intestinal segments in the osteoporosis group, only RPI could be found in the first three intestinal segments. Furthermore, the amounts of baohuoside I and RPI in the osteoporosis group were less than that in the sham group.

The $P^*_{app}$ values of epimedin A in duodenum, jejunum, ileum, and colon ($3.79 \pm 0.18$, $2.72 \pm 0.08$, $0.88 \pm 0.19$, and $0.36 \pm 0.06$, respectively) of the sham group exceeded the values for the osteoporosis group ($2.66 \pm 0.36$, $1.55 \pm 0.16$, $0.41 \pm 0.04$, and $0.17 \pm 0.04$, respectively) (vs. the corresponding bowel, $P < 0.05$). The 10 cm% EP values of epimedin A in the four segments ($69.01 \pm 2.65\%$, $50.30 \pm 2.63\%$, $15.70 \pm 2.45\%$, and $6.36 \pm 1.12\%$, respectively).
respectively) of sham rats was also higher than that of ovariectomized rats (48.61 ± 6.76%, 29.16 ± 3.05%, 7.18 ± 1.70%, and 3.34 ± 0.68%, respectively) (vs. the corresponding bowel, P < 0.05). Under the two condition, from duodenum to colon, the P* app and 10 cm% EP decreased sequentially. The amounts of epimedin A in the four intestinal segments were decreased in turn from duodenum to colon (Figure 6). The amounts of sagittatoside A in the sham group were much greater than that of the osteoporosis group at the relevant intestinal segments.

In sham rats’ duodenum, jejunum, ileum, and colon, the P* app values of epimedin B were 3.52 ± 0.17, 2.44 ± 0.19, 0.60 ± 0.11, and 0.42 ± 0.04, respectively, and the 10 cm% EP values were 66.35 ± 3.15%, 45.18 ± 2.43%, 11.17 ± 2.43%, and 7.83 ± 0.66%, respectively. In osteoporosis rats’ duodenum, jejunum, ileum, and colon, the P* app values of epimedin B were 2.11 ± 0.13, 1.20 ± 0.13, 0.36 ± 0.04, and, 0.23 ± 0.04, respectively, and the 10 cm% EP values were 39.75 ± 3.07%, 23.09 ± 2.47%, 6.91 ± 1.63%, and 4.33 ± 0.73%, respectively. These findings show that for both sham and osteoporosis rats, the P* app of epimedin B in duodenum, jejunum, ileum, and colon fell and, in turn, so did the 10 cm% EP of epimedin B. Compared to the sham group, the P* app and 10 cm% EP values of epimedin B in the osteoporosis group decreased remarkably (vs. the corresponding bowel, P < 0.05). The amount of sagittatoside B was highest in the duodenum and lowest in the colon. The sagittatoside B's content was reduced in osteoporosis group (Figure 7).

In osteoporosis rats’ duodenum, jejunum, ileum, and colon, the P* app values of epimedin C were 1.72 ± 0.11, 1.55 ± 0.12, 0.44 ± 0.06, and 0.28 ± 0.05, respectively, and the 10 cm% EP values were 32.48 ± 2.17%, 26.22 ± 2.36%, 7.90 ± 0.96%, and 4.50 ±
0.90%, respectively. At the corresponding bowel level, the $P_{\text{app}}^*$ and 10 cm% EP values in the sham group were increased significantly compared with the values of the osteoporosis group (vs. the corresponding bowel, $P < 0.05$). The $P_{\text{app}}^*$ values of epimedin C were $3.10 \pm 0.16$, $2.67 \pm 0.11$, $1.02 \pm 0.17$, and $0.48 \pm 0.14$, respectively, and the 10 cm% EP values were $58.47 \pm 3.09\%$, $48.65 \pm 2.06\%$, $17.55 \pm 4.82\%$, and $8.38 \pm 1.36\%$. Sequential reduction was also observed for epimedin C in both sham and osteoporosis groups. The amounts of 2''-O-rahmonosylicarside II in the sham group were higher than that in the osteoporosis group. In the two groups, the amount of 2''-O-rahmonosylicarside II diminished sequentially from duodenum to colon (Figure 8).
Discussion

This study focused on the influence of osteoporosis on absorption and metabolism of epimedium flavonoids. We chose ovariectomized rats as an osteoporotic model. In one series of experiments, we investigated the impact of osteoporosis on hydrolysis of epimedium flavonoids \textit{in vitro} by incubating epimedium flavonoids with intestinal mucosa and feces from osteoporosis and sham rats. In another series, we explored the effect of osteoporosis on the absorption and metabolism of epimedium flavonoids \textit{in vivo} by conducting intestinal perfusion experiments in osteoporosis and sham rats.

With the exception of baohuoside I, whose absorption was unchanged, we found that after oral administration of epimedium flavonoids, most of them were hydrolyzed to secondary glycoside or aglycon by enzymes in the intestine, which led to increased absorption. The enzymes in the intestine have two main sources, intestinal mucosa and intestinal bacteria. When epimedium flavonoids are orally administered, they reach the stomach first. Our earlier studies showed that the main flavonoids in epimedium could not be hydrolyzed by gastric juice (Gao et al., 2013). Hence, the main absorption site of epimedium flavonoids is in the small intestine. In human and rat small intestine (from duodenum to ileum), there are two kinds of $\beta$-glucosidase, cytosolic $\beta$-glucosidase (CBG) and lactose root phlorizin hydrolase (LPH) that could hydrolyze epimedium flavonoids (Nemeth et al., 2003). Both of them could hydrolyze the $\beta$-glycosidic bonds of glycosides, albeit by substantially different hydrolysis mechanisms (Nemeth et al., 2003). For the CBG hydrolysis pathway, because it is an intracellular enzyme, the glycosides must be transported into cells and then hydrolyzed. In our preliminary research, we found that prototypes of epimedium flavonoids had poor membrane
permeability and had a slow uptake by intestinal cells. In addition, it was reported that the transporter of glycoside substances such as SLTGT1 could not transport flavonoids into cells (Dongmei et al., 2012). Therefore, it could be inferred that epimedium flavonoid hydrolysis was not the role of CBG. LPH, a type of extracellular enzyme that is located in the brush border of the small intestinal epithelium, is the only $\beta$-glucosidase in mammalian intestinal brush border. There are two distinct active sites of LPH for catalytic hydrolysis, one for hydrolyzing lactose and flavonoids, and the other for hydrolyzing phlorizin and $\beta$-glucosylceramidase (Tseung et al., 2004). Because LPH is located in the brush border of the small intestinal epithelium (Nemeth et al., 2003), the flavonoids could be hydrolyzed by LPH once they enter the small intestine. Our previous experiments also confirmed that the hydrolysis of epimedium flavonoids decreased after adding LPH inhibitors (Chen et al., 2014), indicating that LPH in intestinal mucosa is the major hydrolase that hydrolyzes epimedium flavonoids. However, the above experiments were carried out on healthy rats, and it was not possible to determine whether the hydrolysis capability of LPH would be different in pathological state.

Because epimedium flavonoids have a good effect against osteoporosis, we studied the hydrolysis capability of LPH in the osteoporotic animals. Our experimental results showed that the hydrolysis rates of epimedium flavonoids in osteoporosis rats was slower than in sham rats, whether the flavonoids were incubated with intestinal enzyme in vitro or were conducted by the in situ intestinal perfusion. These results indicated that the amount and activity of LPH might be lower in osteoporosis rats. Later, the experiment of LPH expression testified our hypothesis that LPH expression was indeed down-regulated in the
ovariectomized rats. Besides, it has been reported that high incidence of fractures and low bone mineral density in menopausal women are correlated with decreased activity and expression of LPH (Bacsi et al., 2007), and the activity of LPH in senile osteoporosis patients is greatly reduced, or even completely inactivated (Bacsi et al., 2007). Both our results and the literatures confirm that low LPH expression is the major factor which decreases the epimedium flavonoid hydrolysis and affects the absorption of flavonoids and their utilization by the body.

In addition to enzymes from intestinal mucosa, enzymes from micro-organisms are involved in the hydrolysis of epimedium flavonoids in the intestinal tract. There were about 100 trillion bacteria in the bowel, of which more than 99% are anaerobic bacteria, including species from the Bacteriodacae, Cattenabacterium, Peptostreptococcus, and Spirillaceal families. Different species of bacteria secrete different metabolic enzymes, and are thereby involved in different types of drug metabolism. For example, there are many bacteria (e.g. Escherichia coli, Enterococcus, etc.) that secrete β-glucosidase, which can hydrolyze β-glucoside bonds for the purpose of detoxification or easier absorption. Epimedium flavonoids that are not fully hydrolyzed in the small intestine travel into the large intestine, where they are for re-absorbed following hydrolysis by bacteria located there. However, owing to the complexity of the intestinal flora, the relationship of intestinal flora and osteoporosis needs to be studied further (Claesson et al., 2012; Koren et al., 2012).

As the body’s largest and most sophisticated micro-ecosystem, maladjusted intestinal bacteria might cause disease, and then the disease could in turn change the function of bacteria (Murphy et al., 2013; Sekirov et al., 2010; Shen et al., 2012; Zhao et al., 2013).
This could explain the reduced hydrolysis rates of icariin, epimedin A, epimedin B, and epimedin C in the intestinal flora of osteoporosis rats, as compared to that observed for sham rats. The above results indicated that intestinal bacteria activity decreased because of the pathological conditions of osteoporosis. Some previous reports in the literature are consistent with our results. It has been reported that the number of anaerobic bacteria, especially *Lactobacillus*, was significantly lower than normal in osteoporosis rats, and the colonization ability of anaerobic bacteria in the intestine was decreased. With respect to metabolism, the ability of bacterial hydrolysis of the glycosidic bonds in general is reduced due to changes in flora and preference for carbon source (Ishihara et al, 2002).

Diseases can alter the hydrolysis capability of intestinal flora and enzymes, which further affects the bioavailability of the drug. Hence, it is necessary to understand the absorption and metabolism of drugs in pathological states. This experiment explored the influence of osteoporosis on the absorption and metabolism of epimedium flavonoids. The results showed that the absorption of epimedium flavonoids was lower in osteoporosis rats than in sham rats. This difference should not be ignored when new therapeutic strategies are developed for the treatment of osteoporosis or some similar diseases. The deficiency of absorption and hydrolysis of epimedium flavonoids in osteoporotic states should be offset, in order for epimedium flavonoids to have a stable anti-osteoporosis efficacy. These results might give rise to new research ideas for similar diseases, providing foundations for deeply exploring the mechanism of absorption and hydrolysis of epimedium flavonoids in osteoporosis rats and for developing new therapeutic strategies in the future.
Conclusions

In this study, we found that the intestinal absorption and hydrolysis of epimedium
flavonoids was slower in osteoporosis rats than in the sham rats. The ovariectomization of
rats resulted in osteoporosis, which might affect the activities of intestinal enzymes, and the
number of intestinal flora. These changes can then affect the intestinal absorption and
hydrolysis of epimedium flavonoids whose structures contain glycosylations.
Authorship Contributions

Participated in research design: Y. Chen, Z. Zhou

Conducted experiments: J. Zhou, Y.H. Ma, Y. Chen, Y. Wang

Contributed new reagents or analytic tools: Y. Wang, X. Gao

Performed data analysis: J. Zhou, Y.H. Ma, Y. Chen

Wrote or contributed to the writing of the manuscript: J. Zhou, Y.H. Ma, Z. Zhou, Y. Chen
References


Footnotes

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J.Z. and Y.H.M contributed equally to this work.
Figure Legends

Figure 1. Chemical structures of five prenylated flavonoids in *Herbal Epimedium* and parts of their metabolites. The symbol “gle” refers to glucose, “rha” to rhamnose, and “xyl” to xylose.

Figure 2. Hydrolysis of epimedium flavonoids after co-incubation with intestinal flora. The x-axis represents incubation time; and the y-axis represents the content of epimedium flavonoids at different time points. Data are mean ± S.D., n = 3.

Figure 3. Hydrolysis behaviors of epimedium flavonoids after co-incubation with intestinal enzymes. The x-axis represents incubation time; and the y-axis represents the content of epimedium flavonoids at different time point. A, B, C, and D represent the content-time profiles of icariin, epimedin A, epimedin B, and epimedin C in intestinal enzyme incubations, respectively. Data are mean ± S.D., n = 3.

Figure 4. Metabolites of epimedium flavonoids after co-incubation with intestinal enzymes. The x-axis represents incubation time; and the y-axis represents relative concentrations of metabolites (the ratio of peak areas of metabolites and testosterone). A, B, C, D, and E represent peak area-time profiles of baohuoside I, RPI, sagittatoside A, sagittatoside B, and 2”'-O-rahamnosylcarnoside II in intestinal enzyme incubations, respectively. Data are mean ± S.D., n = 3.

Figure 5. Absorption and metabolism of icariin in situ intestine model. A. $P_{app}^*$ of icariin in different intestines; B. 10 cm% EP of icariin in different intestines; C. Peak areas of baohuoside I in different intestines; D. Peak areas of RPI in different intestines. Significant differences of $P_{app}^*$ and 10 cm% EP of icariin and amounts of metabolites in different
intestines existed between sham and osteoporosis group (vs. the corresponding bowel, *P < 0.05). Data are mean ± S.D., n = 4.

**Figure 6. Absorption and metabolism of epimedin A in situ intestine model.** A. $P_{\text{app}}^*$ of epimedin A in different intestines; B. 10 cm% EP of epimedin A in different intestines; C. Peak areas of sagittatoside A in different intestines. Significant differences of $P_{\text{app}}^*$ and 10 cm% EP of epimedin A and amounts of sagittatoside A in different intestines existed between sham and osteoporosis group (vs. the corresponding bowel, *P < 0.05). Data are mean ± S.D., n = 4.

**Figure 7. Absorption and metabolism of epimedin B in situ intestine model.** A. $P_{\text{app}}^*$ of epimedin B in different intestines; B. 10 cm% EP of epimedin B in different intestines; C. Peak areas of sagittatoside B in different intestines. Significant differences of $P_{\text{app}}^*$ and 10 cm% EP of epimedin B and amounts of sagittatoside B in different intestines existed between sham and osteoporosis group (vs. the corresponding bowel, *P < 0.05). Data are mean ± S.D., n = 4.

**Figure 8. Absorption and hydrolysis of epimedin C in situ intestine model.** A. $P_{\text{app}}^*$ of epimedin C in different intestines; B. 10 cm% EP of epimedin C in different intestines; C. Peak areas of 2''-O-rahmonosylicarside II in different intestines. Significant differences of $P_{\text{app}}^*$ and 10 cm% EP of epimedin C and amounts of 2''-O-rahmonosylicarside II in different intestines existed between sham and osteoporosis group (vs. the corresponding bowel, *P < 0.05). Data are mean ± S.D., n = 4.
### Table 1 Hydrolysis rates of epimedium flavonoids by intestinal flora

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sham</th>
<th>Osteoporosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Icariin</td>
<td>$\log_{10}C_t = -0.297t + 1.913 \ (R^2 = 0.917)$</td>
<td>$\log_{10}C_t = -0.237t + 1.835 \ (R^2 = 0.952)$</td>
</tr>
<tr>
<td>Epimedin A</td>
<td>$\log_{10}C_t = -0.264t + 1.796 \ (R^2 = 0.974)$</td>
<td>$\log_{10}C_t = -0.212t + 1.832 \ (R^2 = 0.980)$</td>
</tr>
<tr>
<td>Epimedin B</td>
<td>$\log_{10}C_t = -0.270t + 1.756 \ (R^2 = 0.893)$</td>
<td>$\log_{10}C_t = -0.217t + 1.816 \ (R^2 = 0.943)$</td>
</tr>
<tr>
<td>Epimedin C</td>
<td>$\log_{10}C_t = -0.164t + 1.678 \ (R^2 = 0.968)$</td>
<td>$\log_{10}C_t = -0.090t + 1.663 \ (R^2 = 0.900)$</td>
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Table 2 Hydrolysis of epimedium flavonoids by four different intestinal segments for 1 h

<table>
<thead>
<tr>
<th>Compound</th>
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<th>Hydrolysis rates</th>
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<td></td>
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<tr>
<td>Icariin</td>
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<td>Epimedin B</td>
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<tr>
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<tr>
<td>Epimedin C</td>
<td>Osteoporosis</td>
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<td>$R_1$</td>
<td>$R_2$</td>
<td>Compound</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>rha</td>
<td>glc</td>
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<tr>
<td>rha&lt;sup&gt;2&lt;/sup&gt;glc</td>
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<tr>
<td>rha</td>
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<td>Baohuoside I</td>
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<tr>
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</tr>
<tr>
<td>rha&lt;sup&gt;2&lt;/sup&gt;rha</td>
<td>H</td>
<td>2''-O-rahmonosylicarside II</td>
</tr>
</tbody>
</table>
Figure 2

Contents of epimedium flavonoids (%)

Time (h)

Sham

Icariin
Epimedin A
Epimedin B
Epimedin C
Baohuoside I

Osteoporosis

Icariin
Epimedin A
Epimedin B
Epimedin C
Baohuoside I
Figure 8

A

B

C

Sham

Osteoporosis

Sham

Osteoporosis

Sham

Osteoporosis

Duodenum

Jejunum

Ileum

Colon

Duodenum

Jejunum

Ileum

Colon

Duodenum

Jejunum

Ileum

Colon

Duodenum

Jejunum

Ileum

Colon

p<sub>μm</sub>

10 cm% EP

Area (x10<sup>2</sup>)