Special Section on Drug Metabolism and the Microbiome

Intestinal Absorption and Metabolism of Epimedium Flavonoids in Osteoporosis Rats

Jing Zhou, Yi Hua Ma, Zhong Zhou, Yan Chen, Ying Wang, and Xia Gao

ABSTRACT

Herba Epimdii 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 antiosteoporosis activity. To study the medicine in the pathologic 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 hour were reduced by 0.19, 0.26, 0.19, and 0.14, respectively, compared with that in sham rats, which affected the intestinal absorption and hydrolysis of epimedium flavonoids. Their absorption and antiosteoporosis activity were reduced.

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 osteoporosis 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 aged 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).

Herba Epimdii, 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 H. Epimdii were found to be various prenylated epimedium flavonoids (including icarin, epimedin A, epimedin B, epimedin C, and baohuoside I) (Fig. 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 downregulating the expression of DKK1 protein, thereby enhancing bone formation, and they can also prevent ovariectomy-induced bone loss (Zhang et al., 2007; Xu et al., 2011). However, the oral bioavailability of epimedium flavonoids is very low (Wei et al., 2012; Zhou et al., 2013; Chen et al., 2014).

Our previous study found that there are several reasons for this low bioavailability. First, epimedium flavonoids have low absorptive permeabilities due to their physicochemical characteristics (Chen et al., 2008). Second, they are subject to efflux by the drug transporters in intestinal mucosa, such as P-glycoprotein, breast cancer resistance protein, and multidrug resistance-associated protein (Löschner and Potschka, 2005; Chen et al., 2014). Third, these flavonoids can be...
Hydrolyzed by hydrolysis 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 (Zhao et al., 2010; Qian et al., 2012; Cui et al., 2013). The above results were strictly in accordance with the U.S. National Research Council 1996 Guide for the Care and Use of Laboratory Animals and the related ethics regulations of animals.

Therefore, we 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 are revealed, and the differences in 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 U.S. National Research Council 1996 Guide for the Care and Use of Laboratory Animals and the related ethics regulations of Jiangsu Provincial Academy of Chinese Medicine.

Reagents and Chemicals. Icariin (purity >98%) was purchased from the National Institute for the Control of Pharmaceutical and Biologic 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). An enhanced chemiluminescence 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, erythritol, tryptone, and nutrient agar were purchased from Sigma-Aldrich (St. Louis, MO). All other materials (typically analytical grade or better) were used as received.

Animals. Eight-week-old female Sprague-Dawley rats with a body weight of 170–250 g were obtained from the Shanghai Laboratory 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: the sham group (sham) or the ovariectomized group (osteoporosis). After 1 week of acclimatization, rats were anesthetized with an intraperitoneal injection of 300 mg chloral hydrate per 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. Bone mineral density measurements of the femur in rats confirmed that the osteoporosis model was available for subsequent experiments.

Intestinal Flora Hydrolysis of Epimedium Flavonoids. Anaerobic culture medium was prepared as follows: K2HPO4 [37.5 ml, 0.78%], solution A [37.5 ml, 0.47% KH2PO4, 1.18% NaCl, 1.2% (NH4)2SO4, 0.12% CaCl2, and 0.25% MgSO4·H2O], Na2CO3 [50 ml, 8%], L-cysteine [0.5 g], l-ascorbic acid [2 ml, 25%], erythritol [1 g], tryptone [1 g], and nutrient agar [1 g] were mixed together and diluted with distilled water to 1 liter. The solution was then adjusted to pH 7.5–8.0 with 2 M HCl (Akao et al., 1996).

Fresh feces collected from Sprague-Dawley rats was immediately homogenized in normal saline solution at a ratio of 1 g to 4 ml. The homogenate was filtered, and the filtrate of fresh feces (10 ml) was added to 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) was 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 hours at 37°C, the incubations were deproteinized by adding a 3-fold volume of acetonitrile. The samples were vortexed for 1 minute and centrifuged for 15 minutes at 15,000 rpm. For high-performance liquid chromatography (HPLC)-UV analysis, 400-μl aliquots of the supernatants were mixed with 100 μM internal standard testosterone (100 μl), which was dissolved in acetonitrile. For ultra performance liquid chromatography (UPLC)/quadrupole time-of-flight mass spectrometry (Q-TOF-MS) analysis, the supernatants (150 μl) were purified using a C18 solid phase extraction cartridge (Agilent, Santa Clara, CA). The cartridge was activated by adding a 3-fold volume of acetonitrile. The samples were vortexed for 1 minute and centrifuged for 15 minutes at 15,000 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, and fresh feces was collected from both groups at 3 months postoperation.

Intestinal Enzyme Hydrolysis of Epimedium Flavonoids. After overnight food deprivation, rats were anesthetized by an 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 dividing the duodenum, jejunum, ileum, and colon into segments, the contents of the four segments were removed by gently flushing them with saline (0°C). Intestinal mucosa was blunted scratched. Intestinal mucosa in different segments were immediately homogenized and centrifuged at 13,000 rpm for 15 minutes. Supernatants (150 μl) 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, and fresh feces was collected from both groups at 3 months postoperation.

![Chemical structures of five prenylated flavonoids in H. Epimediti and parts of their metabolites.](image)

![Fig. 1.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sham Group</th>
<th>Osteoporosis Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LogCt</td>
<td>R²</td>
</tr>
<tr>
<td>Icariin</td>
<td>-0.297 ± 1.913</td>
<td>0.917</td>
</tr>
<tr>
<td>Epimedin A</td>
<td>-0.264 ± 1.796</td>
<td>0.974</td>
</tr>
<tr>
<td>Epimedin B</td>
<td>-0.270 ± 1.756</td>
<td>0.893</td>
</tr>
<tr>
<td>Epimedin C</td>
<td>-0.164 ± 1.678</td>
<td>0.968</td>
</tr>
</tbody>
</table>

Cl, threshold cycle.
segments was immediately homogenized in normal saline solution at a 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 (Sesink et al., 2003).

The subsequent procedures and animal group assignment were the same as the experiments of intestinal flora-hydrorlyzing epimedium flavonoids. At the same time, protein was extracted from frozen intestines by radioimmunoprecipitation assay buffer containing 1 mM phenylmethylsulfonyl fluoride for 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 a silk suture. The intestine was carefully arranged and continuously monitored to avoid kinks, and a consistent flow was ensured after cannulation. Saline-soaked cotton was used to cover opened body cavities to prevent loss of fluids (Andlauer et al., 2000a,b,c; Liu and Hu, 2002; Chen et al., 2003; Higaki et al., 2004).

After 3-month ovarectomized 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 minutes. 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 that of 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 the intestinal flora and enzyme samples. Therefore, HPLC-UV was used for determining the contents of epimedium flavonoids in intestinal flora and enzyme samples, whereas UPLC-UV was used for determining the contents of epimedium flavonoids in perfusate samples. All of the metabolites were identified by the UPLC/Q-TOF-MS method.

The UPLC-UV method was performed with an Acquity UPLC system with a photodiode array detector and Empower software (Waters, Milford, MA) and an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm). The mobile phase consisted of acetonitrile (A) and water (B), with the following gradient program: 0 to 0.5 minutes, 25% A; 0.5 to 1.5 minutes, 25% to 28% A; 1.5 to 2.5 minutes, 28% A to 32%; 2.5 to 3.5 minutes, 32% A to 35%; 3.5 to 4.5 minutes, 35% A to 55%; 4.5 to 5.5 minutes, 55% A to 95%; and 5.5 to 6.0 minutes, 95% A. The flow rate was 0.4 ml/min, the column temperature was 30°C, and the injection volume was 5 μl. The wavelength was 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 of less than 2%. The accuracy and precision were greater than 97%. The tested linear response ranges for all flavonoids were 6.25–100 μM, respectively. Epimedium flavonoids exhibited good linearity within the selected concentration ranges, with R² values between 0.9992 and 0.9997.

The UPLC/Q-TOF-MS method was performed with a Waters Synapt G2-S Q-TOP mass spectrometer equipped with an electrospray ionization source. The samples were separated on an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm; Waters). The mobile phase consisted of acetonitrile (A) and water (B). The gradient program of epimedium flavonoids was as follows: 0 to 1 minute, 5% A; 1 to 1.5 minutes, 5% to 25% A; 1.5 to 3 minutes, 25% A; 3 to 4 minutes, 25% to 28% A; 4 to 5 minutes, 28% to 32% A; 5 to 6 minutes, 32% to 35% A; 6 to 7 minutes, 35% to 55% A; 7 to 8 minutes, 55% to 95% A; 8 to 9.5 minutes, 95% A; and 9.5 to 10 minutes, 95% to 5% A. The flow rate was 0.4 ml/min, the column temperature was maintained at 30°C, and the injection volume was 2 μl. The tested linear response ranges for samples were 1.25–40 μM. Epimedium flavonoids exhibited good linearity within the selected concentration ranges, with R² values between 0.9992 and 0.9996.

For mass spectrometry analysis, the electrospray ionization source was operated in the positive ion mode. The capillary and cone voltages were 3000 and 20 V, respectively. The tapered cone 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 was as follows: low energy, 6 V; and 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 a mass-to-charge ratio (m/z) of 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 epimediin 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 epimediin 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 epimediin 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. To analyze the hydrolysis behavior of epimedium flavonoids in intestinal flora and enzyme incubation, the hydrolysis rate of epimedium flavonoids was calculated using the following equation:

$$Hydrolysis \ rate = \frac{A_t - A_i}{A_i}$$

(1)

Fig. 2. Hydrolysis of epimedium flavonoids after 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 means ± S.D. (n = 3).
In eq. 1, \( A_t \) is the total amount of epimedium flavonoids before hydrolysis, and \( A_r \) is the residual amount of epimedium flavonoids after hydrolysis.

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

\[
P_{\text{app}}^* = -\frac{v}{2\pi rl} \ln \frac{V_{\text{out}}}{V_{\text{in}}} \frac{r_{\text{out}}}{r_{\text{in}}}
\]

In eq. 2, \( V_{\text{in}} \) and \( V_{\text{out}} \) (in milligrams) are volumes of poured and collected testing solution, respectively; \( v \) is the perfusion velocity; \( r_{\text{out}} \) and \( r_{\text{in}} \) (in grams per liter) are concentrations of inlet and outlet solutions, respectively; \( l \) (in centimeters) is the length of the perfused bowel (typical length is 10 cm); and \( r \) (in centimeters) is the cross-sectional radius of the perfused bowel. Values are indicated as means \( \pm \) S.D.

Statistical Analysis. The \( t \) test (Microsoft Excel; Microsoft Corporation, Redmond, WA) was used to analyze the data. The prior level of 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 rapidly hydrolyzed.

Fig. 3. Hydrolysis behaviors of epimedium flavonoids after coincubation with intestinal enzymes. The x-axis represents incubation time, and the y-axis represents the content of epimedium flavonoids at different time points. (A–D) Content-time profiles of icariin, epimedin A, epimedin B, and epimedin C in intestinal enzyme incubations, respectively. Data are means \( \pm \) S.D. (n = 3).
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, hydrolysis of icariin by the sham group was completed at 1.5 hours, whereas hydrolysis in the osteoporosis group was not complete until 2 hours. The hydrolysis rates of epimedin A after 1-hour 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 were 0.30, 0.16, 0.21, and 0, respectively, for the sham group and 0.22, 0.090, 0, and 0, respectively, for the osteoporosis group. For epimedin C, the rates were 0.63, 0.70, 0.090, and 0, respectively, for the sham group and 0.32, 0.23, 0.070, and 0, respectively, for the osteoporosis group.

The metabolites generated were also measured to evaluate the hydrolysis capability of intestinal enzymes. As shown in Fig. 4, the amounts of metabolites of epimedium flavonoids incubated with four different intestinal segments were higher in the sham rats than in the osteoporosis rats. Metabolites of icariin, baohuoside I, and RPI were detected after incubation with enzymes from the 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 that RPI might be transferred to baohuoside I over time. Epimedin A, epimedin B, and epimedin C have similar structures, suggesting that their hydrolysis mechanisms might be similar. Their metabolites, sagittatoside A, sagittatoside B, and 2’’-O-rahmosylsikarside 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 after hydrolysis by intestinal enzymes, such as LPH, and then absorbed as metabolites. The apparent permeability coefficient (P_{app}) and eliminative percentage (10 cm% 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 enzymes (Supplemental Fig. 2), further confirming

### TABLE 2

Hydrolysis of epimedium flavonoids by four different intestinal segments for 1 hour

<table>
<thead>
<tr>
<th>Compound</th>
<th>Group</th>
<th>Hydrolysis Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duodenum</td>
<td>Jejunum</td>
</tr>
<tr>
<td>Icariin</td>
<td>Sham</td>
<td>0.89</td>
</tr>
<tr>
<td>Epimedin A</td>
<td>Sham</td>
<td>0.39</td>
</tr>
<tr>
<td>Epimedin B</td>
<td>Sham</td>
<td>0.15</td>
</tr>
<tr>
<td>Epimedin C</td>
<td>Sham</td>
<td>0.63</td>
</tr>
</tbody>
</table>

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**References:**

Fig. 4. Metabolites of epimedium flavonoids after coincubation 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–E) Peak area-time profiles of baohuoside I, RPI, sagittatoside A, sagittatoside B, and 2′′-O-rahmonosylicarside II in intestinal enzyme incubations, respectively. Data are means ± S.D. (n = 3).
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 Figs. 5–8. As shown in Fig. 5, in both groups, the $P^\text{app}$ and 10 cm% EP values of icariin became progressively smaller from the duodenum to the colon. In the sham group, the $P^\text{app}$ values of icariin in the duodenum, jejunum, ileum, and colon were 5.59 ± 0.57, 5.26 ± 0.74, 1.30 ± 0.18, and 0.50 ± 0.09, respectively, and the 10 cm% EP values of icariin were 72.26% ± 7.89%, 69.09% ± 8.29%, 19.66% ± 2.93%, and 9.48% ± 1.50%, respectively. For the osteoporosis group, the $P^\text{app}$ values of icariin in the duodenum, jejunum, ileum, and colon were 3.87 ± 0.46 (duodenum), 3.57 ± 0.55 (jejunum), 0.73 ± 0.60 (ileum), and 0.36 ± 0.29 (colon), and the 10 cm% EP values of icariin were 50.20% ± 5.34%, 44.17% ± 6.18%, 9.01% ± 1.35%, and 4.72% ± 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 in the osteoporosis group, and 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 $P^\text{app}$ values of epimedin A in the duodenum, jejunum, ileum, and colon (3.79 ± 0.18, 2.72 ± 0.08, 0.88 ± 0.19, and 0.36 ± 0.06, respectively) of the sham group exceeded the values for the osteoporosis group (2.66 ± 0.36, 1.55 ± 0.16, 0.41 ± 0.04, and 0.17 ± 0.04, respectively) (versus the corresponding bowel, $P < 0.05$). The 10 cm% EP values of epimedin A in the four segments (69.01% ± 2.65%, 50.30% ± 2.63%, 15.70% ± 2.45%, and 6.36% ± 1.12%, respectively) of sham rats were 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) (versus the corresponding bowel, $P < 0.05$). Under the two conditions, from the duodenum to the colon, the $P^\text{app}$ and 10 cm% EP decreased sequentially. In turn, the amounts of epimedin A in the four intestinal segments were decreased from the duodenum to the colon (Fig. 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 $P^\text{app}$ 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 $P^\text{app}$ 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 $P^\text{app}$ of epimedin B in the duodenum, jejunum, ileum, and colon fell and, in turn, so did the 10 cm% EP of epimedin B. Compared with the sham group, the $P^\text{app}$ and 10 cm% EP values of epimedin B in the osteoporosis group decreased remarkably (versus the corresponding bowel, $P < 0.05$). The amount of sagittatoside B was highest in the duodenum and lowest in the colon. The sagittatoside B content was reduced in the osteoporosis group (Fig. 7).

In osteoporosis rats’ duodenum, jejunum, ileum, and colon, the $P^\text{app}$ 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 significantly increased compared with the values of the osteoporosis group (versus 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 the 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 the duodenum to the colon (Fig. 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 effect of osteoporosis on hydrolysis of epimedium flavonoids 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 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 intestines (from the duodenum to the ileum), there are two kinds of $\beta$-glucosidase that could hydrolyze epimedium flavonoids: LPH and cytosolic $\beta$-glucosidase (CBG) (Németh et al., 2003). Both could hydrolyze the $\beta$-glycosidic bonds of glycosides, albeit by substantially different hydrolysis mechanisms (Németh et al., 2003). For the CBG hydrolysis pathway, the glycosides must be transported into cells and then hydrolyzed, because CBG is an intracellular enzyme. 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 the 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 $\beta$-glucosylceramide (Tseung et al., 2004). Because LPH is located in the brush border of the small intestinal epithelium (Németh 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 in healthy rats, and it was not possible to determine whether the hydrolysis capability of LPH would be different in a pathologic state.
Because epimedium flavonoids have a strong 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 enzymes in vitro or were conducted by 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 affirmed our hypothesis that LPH expression was indeed downregulated in the ovariectomized rats. In addition, it has been reported that the 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 literature confirm that low LPH expression is the major factor that decreases epimedium flavonoid hydrolysis and affects the absorption of flavonoids and their utilization by the body.

In addition to enzymes from intestinal mucosa, enzymes from microorganisms are involved in the hydrolysis of epimedium flavonoids in the intestinal tract. There are about 100 trillion bacteria in the bowel, of which more than 99% are anaerobic bacteria, including species from the Bacteriodaceae, Erysipelotrichaceae (Catenibacterium), Clostridiaceae (Peptostreptococcus), and Spirillaceae 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 reabsorbed after hydrolysis by bacteria located there. However, owing to the complexity of the intestinal flora, the relationship of intestinal flora and osteoporosis needs to be further studied (Claesson et al., 2012; Koren et al., 2012).

As the body’s largest and most sophisticated microecosystem, maladjusted intestinal bacteria might cause disease, and the disease could in turn then change the function of bacteria (Sekirov et al., 2010; Murphy et al., 2013; Shen et al., 2013; 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 compared with that observed for sham rats. The above results indicated that intestinal bacteria activity decreased because of the pathologic 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 hydrolytic capability of intestinal flora and enzymes, which further affects drug bioavailability. Hence, it is necessary to understand the absorption and metabolism of drugs in pathologic 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 similar diseases. The deficiency of absorption and hydrolysis of epimedium flavonoids in osteoporotic states should be offset, in order for epimedium flavonoids to have...
stable antiosteooporosis 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 sham rats. 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: Chen, Z. Zhou.
Conducted experiments: J. Zhou, Ma, Chen, Wang.
Contributed new reagents or analytic tools: Wang, Gao.
Performed data analysis: J. Zhou, Ma, Chen.
Wrote or contributed to the writing of the manuscript: J. Zhou, Ma, Z. Zhou, Chen.

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Fig. 8. Absorption and hydrolysis of epimedin C in situ intestine model. (A) P* app of epimedin C in different intestines. (B) 10 cm% EP of epimedin C in different intestines. (C) Peak areas of 2'-O-rahmonosylicarlside II in different intestines. Significant differences of P* app and 10 cm% EP of epimedin C and amounts of 2'-O-rahmonosylicarlside II in different intestines existed between the sham and osteoporosis groups (versus the corresponding bowel, *P* < 0.05). Data are means ± S.D. (n = 4).
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