**Intestinal Absorption and Metabolism of Epimedium Flavonoids in Osteoporosis Rats**

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**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 glycodies 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. Hydrolysis rates were reduced by 0.21, 0.24, 0.08, and 0.31 for icariin, epimedin A, epimedin B, and epimedin C incubated with duodenal enzymes for 1 hour and by 0.13, 0.09, 0.07, and 0.47 for icariin, epimedin A, epimedin B, and epimedin C incubated with jejunum enzymes, respectively, compared with the sham group. In addition, the apparent permeability coefficient and elimination percentage of the four epimedium flavonoids in the duodenum, jejunum, ileum, and colon decreased by 29%, 56%, and 50%–56%, and 27%–53% compared with 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 enzymes changed in ovariectomized rats, which affected the intestinal absorption and hydrolysis of epimedium flavonoids whose structures contain 7-glucose.

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**Introduction**

Osteoporosis is characterized by the reduction and deterioration of the bone microarchitectural, 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 icariin, 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...
Absorption and Metabolism of Epimedium Flavonoids

FIG. 1. Chemical structures of five prenylated flavonoids in H. Epimdii and parts of their metabolites. gluc, glucose; rha, rhamnose; xyl, xylose.

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>Icarin</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>Baohuoside I</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–hydrolyzing epimedium flavonoids. At the same time, protein was extracted from frozen intestines by radioimmunoprecipitation assay buffer containing 1 mM phenylmethylsulfonylfluoride 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 the 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 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 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. 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 Agilent 1260 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 water (A) and acetonitrile (B), with the following gradient program: 0 to 6.5 minutes, 70% A; 6.5 to 10 minutes, 45% A; 10 to 14 minutes, 45% A; and 14 to 15 minutes, 70% A. The flow rate was 1 ml/min and the injection volume was 10 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-Q-TOF 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 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 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 513.07 and 313.07. 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. 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:

\[
\text{Hydrolysis rate} = \frac{A_i - A_f}{A_i}
\]

Fig. 2. Hydrolysis of epimedium flavonoids after coincubation 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 ($P_{app}^*$) was determined through the rate of drug disappearance. The $P_{app}^*$ was calculated using the following equation:

$$P_{app}^* = -v \times \frac{1}{2\pi rl} \ln \frac{V_{out}}{V_{in}} \rho_{out} \rho_{in}$$

In eq. 2, $V_{in}$ and $V_{out}$ (in milligrams) are volumes of poured and collected testing solution, respectively; $v$ is the perfusion velocity; $\rho_{out}$ and $\rho_{in}$ (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 ± 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.
HPLC-UV analysis demonstrated that each of the flavonoids had only one metabolite (Supplemental Figs. 1 and 2). UPLC/Q-TOF-MS analysis 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-rhamnosyliscaraside 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 in sham rats (slope values = 0.297, 0.264, 0.279, and 0.164, respectively) 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 incubation for 1 hour 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, epimedin A, epimedin B, and epimedin C was 0.56, 0.37, 0.40, and 0.37, respectively. Icariin, epimedin A, and epimedin B could not be detected after 4 hours 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 hours. The hydrolysis of epimedin C was slower than the other three compounds and it could still be detected after 8 hours 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 (Fig. 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 Fig. 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). These results indicate that hydrodase might be different in intestinal feces and mucosa.

The hydrolysis rates of epimedium flavonoids incubated with four different intestinal segments from sham and osteoporosis rats were calculated. As shown in Fig. 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-hour 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, 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, 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, 0.070, 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-rhamnosyliscaraside 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 (Papp) 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>
<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></td>
<td>Osteoporosis</td>
<td>0.68</td>
</tr>
<tr>
<td>Epimedin A</td>
<td>Sham</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>Osteoporosis</td>
<td>0.15</td>
</tr>
<tr>
<td>Epimedin B</td>
<td>Sham</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>Osteoporosis</td>
<td>0.22</td>
</tr>
<tr>
<td>Epimedin C</td>
<td>Sham</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>Osteoporosis</td>
<td>0.32</td>
</tr>
</tbody>
</table>
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-rahmnosylarctiside 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 fell and, in turn, so did the 10 cm% EP of icariin in the four intestinal segments. Furthermore, the amounts of baohuoside I and RPI of icariin in different intestines existed between the sham and osteoporosis groups (versus the corresponding bowel, *$P < 0.05$). Data are means ± S.D. ($n = 4$).

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 sagittatose B was highest in the duodenum and lowest in the colon. The sagittatose B content was reduced in the osteoporosis group (Fig. 6).

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. The amount of sagittatose B was highest in the duodenum and lowest in the colon. The sagittatose B content was reduced in the osteoporosis group (Fig. 7).
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.

Sequentially, 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 $\beta$-glucosylceramidase (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 antiosteoporosis 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. Ovariecotomization 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 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) Papp of epimedin C in different intestines. (B) 10 cm% EP of epimedin C in different intestines. (C) Peak areas of 2'-O-rhamnosylsaccharide II in different intestines. Significant differences of Papp and 10 cm% EP of epimedin C and amounts of 2'-O-rhamnosylsaccharide 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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