Introduction

Previous epidemiological data showed that a lower prevalence (5-6%) of osteonecrosis (ON) was found in patients recovered from SARS frequently prescribed with a crude extract of flavonoids rich "Bone Strengthening Chinese Herb" Epimedium during their rehabilitation in southern China1,2, whereas a higher prevalence (32.7%) of ON was found in those seldom prescribed with a crude extract of Epimedium in northern China3. Recently, using a small scale laboratory isolation procedure, we reported that flavonoids derived from "the Bone Strengthening Chinese Herb" Epimedium demonstrated a beneficial effect on prevention of steroid-associated ON with inhibition of both intravascular thrombosis and extravascular lipid-deposition in an established rabbit model based on a single dose study design4,5. Now, a simplified procedure for isolating flavonoids from herbal Epimedium, i.e., Epimedium-derived Flavonoids (EF) to meet the requirement of large scale production has been established, which generates seven major phytoestrogenic flavonoid compounds with common stem nuclear character-

Materials and methods

The present study used sixty-two 28-week-old male New-Zealand white rabbits and an established injection protocol for inducing steroid-associated ON4,5. Apart from the control group (CON; n=14; corresponding vehicle/day), rabbits were assigned into the following groups for receiving simultaneous oral administration of EF, including a low dose group (L-EF: n=16; 10 mg/kg body weight/day), a middle dose group (M-EF: n=16; 20 mg/kg/day), and a high dose group (H-EF: n=16; 40 mg/kg/day). Two weeks later after induction, bilateral proximal femora were dissected for MicroCT-based angiography of intraosseous vasculature and histopathological examination of ON lesions and size distribution of extravascular leakage particles. The incidence of ON was defined as the numbers of ON rabbits divided by the numbers of total rabbits in each group. All corresponding evaluation protocols were established in our previous studies4,5.

Results

The results showed that the incidence of ON in the L-EF group (9/16, 56%), M-EF group (2/16, 13%) and H-EF group (6%) was significantly lower than that in the CON group (13/14, 93%), respectively. Furthermore, the incidences of ON in the M-EF group (2/16, 13%) and H-EF group (6%) were also significantly lower than that in the L-
EF group (9/16, 56%), whereas there was no significant difference in ON incidence between the M-EF group (2/16, 13%) and the H-EF group (6%) (Figure 2).

The angiograms in the CON group at week 1 post-induction showed dilated vessel-like structural units (>600 μm) surrounded by both numerous middle sized disseminated leakage-particle-like structural units (200 ~ 400 μm) and few vessel-like structural units (36 ~ 200 μm or 400 ~ 600 μm). Relative to those in the CON group at week 1 post-induction, no typical dilated vessel-like structural units surrounded by numerous middle size disseminated leakage-particle-like structural units (200 ~ 400 μm) were found in both the M-EF group and the H-EF group except for a few in the L-EF group, and reduced perfusion to vessel-like structural units (36 ~ 200 μm or 400 ~ 600 μm) were rarely found in both the M-EF group and the H-EF group but moderate in the L-EF group (Figure 3). Extravascular leakage particles during perfusion for MicroCT-based angiography were found more in the CON group after induction, whereas they were rarely found in both the M-EF and H-EF groups except for a few in the L-EF group after induction. All the histograms showed that the size of the leakage particles ranged between 200 μm and 400 μm (Figure 4).

**Discussion and conclusion**

Structurally, the results of MicroCT-based angiography suggested a prominent decrease in perfused vessels, which was evidenced by the presence of few vessel-like structural units

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Retention Time (min)</th>
<th>Name</th>
<th>Molecular Form</th>
<th>Common Structure</th>
<th>Area (%)</th>
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<tr>
<td>1</td>
<td>7.9</td>
<td>Epimedeside A</td>
<td>C_{32}H_{38}O_{15}</td>
<td>α-L-Rha Glc H</td>
<td>1.8</td>
</tr>
<tr>
<td>2</td>
<td>16.0</td>
<td>Hexandraside F</td>
<td>C_{39}H_{50}O_{20}</td>
<td>4-O-(β-D-Glc)-α-L-Rha Glc CH_{3}</td>
<td>1.2</td>
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<tr>
<td>3</td>
<td>18.4</td>
<td>Epimedin A</td>
<td>C_{39}H_{50}O_{20}</td>
<td>2-O-(β-D-Glc)-α-L-Rha Glc CH_{3}</td>
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</tr>
<tr>
<td>4</td>
<td>20.3</td>
<td>Epimedin B</td>
<td>C_{38}H_{48}O_{19}</td>
<td>2-O-(β-D-Xyl)-α-L-Rha Glc CH_{3}</td>
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<tr>
<td>5</td>
<td>22.7</td>
<td>Epimedin C</td>
<td>C_{39}H_{50}O_{19}</td>
<td>2-O-(α-L-Rha)-α-L-Rha Glc CH_{3}</td>
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</tr>
<tr>
<td>6</td>
<td>24.5</td>
<td>Icarin</td>
<td>C_{33}H_{46}O_{15}</td>
<td>α-L-Rha Glc CH_{3}</td>
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<tr>
<td>7</td>
<td>55.7</td>
<td>Baohuoside-I</td>
<td>C_{27}H_{30}O_{10}</td>
<td>α-L-Rha H CH_{3}</td>
<td>2.1</td>
</tr>
</tbody>
</table>

**Table 1.** Seven main flavonoid molecules identified in Epimedium-derived Flavonoids.
(36 ~ 200 µm or 400 ~ 600 µm) excluding leakage-particle-like structural units (200 ~ 400 µm) at week 1 post-induction compared to those of intact sample (our previous work). Furthermore, the reduced perfusion to vessel-like structural units (36 ~ 200 µm or 400 ~ 600 µm) was hardly found in both the M-EF group and H-EF group but moderate in the L-EF group at week 1 post-induction. It suggested that Epimedium-derived Flavonoids (EF) exerted beneficial effects on maintaining intraosseous vasculature. Histologically, the results from histopathological examination showed EF exerted beneficial effects on inhibiting the incidence of steroid-associated ON in a dose-dependent manner in rabbits. Accordingly, our
findings implied that the beneficial effect of EF on reducing the risk of steroid-associated ON could be explained by its maintaining intraosseous vasculature in a dose-dependent manner in the rabbit model.

Acknowledgements

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References


Figure 4. Representative histology images of particles (indicated by arrow) were extravascularly distributed in CON group at week 1 post-induction (A), whereas particles were intravascularly distributed in the M-EF group at week 1 post-induction (B).