Protective effect of puerarin form the roots of *Pueraria lobata* against systemic inflammatory response syndrome by regulating the levels of related cytokines

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The outcome of systemic inflammatory response syndrome (SIRS) is poor due to unclear pathogenesis and unsatisfied therapeutic strategies. Therefore, to understand the pathogenetic mechanism and screen novel drugs are critical for the improvement of the therapeutic efficacy of SIRS. In the present study, to determine the protective mechanism of puerarin on SIRS, Sprague Dawley (SD) rats were intraperitoneally injected with different doses of zymosan-A to generate an experimental SIRS animal model. The peripheral TNF-α, IL-6 and IL-10 levels of SIRS rats were measured using quantitative ELISA assay. Protective effects puerarin on SIRS rats via regulating cytokine levels was subsequently determined. The results showed about 75.0% of SIRS rats died after injection with 1000 mg/kg zymosan-A, whereas only 16.7% of SIRS rats (1000 mg/kg zymosan-A) were found dead after the treatment with 62.5 mg/kg puerarin (P< 0.01 vs SIRS group). 24 h after injection with 750 mg/kg zymosan-A, the peripheral levels of TNF-α, IL-6 and IL-10 were 30.87±6.81 pg/ml, 525.20±92.45 pg/ml and 1.37±0.17 ng/ml, respectively. However, the levels of TNF-α and IL-6 (16.71±3.75 pg/ml and 399.30±77.87 pg/ml, respectively) were significantly lowered (P< 0.01) and IL-10 level (1.95±0.17 ng/ml) was markedly elevated (P< 0.01) after treatment with 62.5 mg/kg puerarin when compared with those in SIRS group. Puerarin conferred protective effects on the SIRS via down-regulating pro-inflammatory TNF-α and IL-6 as well as up-regulating anti-inflammatory IL-10.

Key words: Experimental SIRS, puerarin, protective effects, cytokines.

INTRODUCTION

Systemic inflammatory response syndrome (SIRS) is a severe and acute disease commonly found in human beings and it’s mainly caused by either infectious or non-infectious agents (Baue et al., 1998; Baue, 2000). The disease easily develops to multiorgan dysfunction syndrome (MODS) or multiple organ failure (MOF) resulting in about 70% mortality (Werdan et al., 2009; Hoesel and Ward, 2004). Favorable therapeutic outcome is seldom obtained in clinical practice due to the complicated contributing factors, unclear pathogenesis and unsatisfied therapeutic strategies (Baue et al., 1998; Luo et al., 2005; Konkel et al., 2008).

Puerarin is a compound derived from roots of *Pueraria thomsonii* and *Pueraria lobata*, and its main active substance has been identified as 8-β-D-glucopyranose-4, 7-dioxyisoflavonoid glycoside (Parthasarathy and Santanam, 1994). Puerarin has extensive pharmaceutical effects as an antioxidant and β-adrenoreceptor blocker (Abenavoli, 2009; Chen et al., 1995; Ji and Wang, 1996; Dong and Wang, 1998; Xu et al., 2007; Chang et al., 2009).
In China, puerarin has been used as a traditional medicine for treating various diseases including cardiovascular disorders and cerebrovascular diseases for more than ten years (Chang et al., 2009; Fan et al., 1992; Wang et al., 1997; Xuan et al., 1999; Wang et al., 2008; Xu and Zhao, 2002). Furthermore, it was reported that puerarin could regulate inflammatory cytokines in brain and heart of animal models following ischemia-reperfusion (Yang and He, 2003; Zhu and Yao, 2001). Previous studies revealed that numerous cytokines played critical roles in the occurrence and development of SIRS, among which, pro-inflammatory factors such as TNF-α and IL-6 as well as anti-inflammatory factor including IL-10 have been confirmed to be the most important (Netea et al., 2003; Miyakawa et al., 2005; Ferrer et al., 1998; Riewald and Ruf, 2003; Ely et al., 2003). However, till date, little has been known about the therapeutic effects of puerarin on SIRS.

In the present study, rat SIRS model was used to determine the protective effects of puerarin on SIRS. To further understand the potential mechanisms underlying the protective effects of puerarin on experimental SIRS, the peripheral levels of TNF-α, IL-6 and IL-10 of SIRS rats were determined before and after administration of puerarin. This study will provide a novel strategy for the treatment of SIRS in clinical practice.

MATERIALS AND METHODS

Animals

Five to seven week-old Sprague-Dawley (SD) rats weighing 160 - 200 g were used with a male to female ratio of 1:1. The rats were housed in a temperature-controlled room (25 °C) and given free access to food and water. All animal experiments and care were performed according to the guide for the care and use of laboratory animals and the whole protocol was approved by our university.

Reagents

The puerarin solution (100 mg/2 ml) was supplied by Zhejiang CONBA Pharmaceutical Co. Ltd. (Zhejiang, China), and the chemical structure provided by the manufacturer was shown in Figure 1. Zymosan-A and liquid paraffin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA)—kits of TNF-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were purchased from R&D Systems Inc. (Minneapolis, MN, USA).

Establishment of the SIRS rat model

Zymosan-A powder and liquid paraffin were mixed, followed by high frequency oscillation for 15 min. Then, the zymosan-A-paraffin suspension (ZPS) was sterilized at 100 °C water-bath for 80 min and stored at 4 °C (Werdan et al., 2009; Hoesel and Ward, 2004). Before use, ZPS was pretreated with high frequency oscillation for 15 min in 40 °C water-bath. 18 h before the experiment, the rats were fasted and given access to water ad libitum. 2 h before the experiment, the weight and rectal temperature of rats were measured. The animals were randomly divided into four groups (n = 12 in each group). The rats in Group A, B and C were intraperitoneally injected with zymosan-A of different concentrations (500, 750 and 1000 mg/kg, respectively), and those in Group D (control group) were treated with sterilized normal saline of equal volume. Then, all animals were routinely given access to water and food ad libitum during the following days. The diagnostic criteria for SIRS in rats was described as the follows: the rectum temperature of treated rats was increased or decreased by 1 °C and the number of white blood cells (WBC) was increased by two fold or decreased by 50% (Werdan et al., 2009; Hoesel and Ward, 2004). Caudal vein blood was collected for WBC counting which was performed with an automatic blood cell analyzer (STKS type, Beckman Coulter, USA).

Protective test of puerarin in SIRS rats

Thirty-six SD rats were randomly divided into three groups (n = 12 in each group): Rats in Group A were intraperitoneally injected with 1000 mg/kg zymosan-A, those in Group B were administrated with 62.5 mg/kg puerarin through caudal vein immediately after injection of 1000 mg/kg zymosan-A, and those in Group C were intraperitoneally injected with sterilized normal saline of equal volume as the control. All the animals were routinely given free access to water and food during the following days and survival rate was detected. The ZPS pre-treatment, animal starving, weighing and rectum temperature measuring, and diagnostic criteria of SIRS were the same as described previously.

Detection of SIRS-related cytokines

Thirty-six SD rats were randomly divided into three groups (n = 12 in each group): Rats in Group A were intraperitoneally injected with 750 mg/kg zymosan-A, those in Group B were injected with 62.5 mg/kg puerarin through caudal vein immediately after injection of 750 mg/kg zymosan-A, and those in Group C were intraperitoneally injected with the sterilized normal saline of equal volume as the control. 24 h after injection, caudal vein blood were collected for the detection of TNF-α, IL-6 and IL-10 levels with commercial ELISA.

Figure 1. Basic chemical constitution of puerarin used in this study.
Table 1. Mortality, temperature and total WBC in rats injected with zymosan-A.

<table>
<thead>
<tr>
<th>Group</th>
<th>Zymosan-A (mg/kg)</th>
<th>Cases (n)</th>
<th>Survival (n)</th>
<th>Temperature (°C)</th>
<th>Total WBC (×10^3/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td>A</td>
<td>500</td>
<td>12</td>
<td>12</td>
<td>37.2±0.9</td>
<td>36.9±0.8</td>
</tr>
<tr>
<td>B</td>
<td>750</td>
<td>12</td>
<td>12</td>
<td>37.1±0.8</td>
<td>36.1±1.1</td>
</tr>
<tr>
<td>C</td>
<td>1000</td>
<td>12</td>
<td>2</td>
<td>37.0±1.0</td>
<td>35.6±1.1</td>
</tr>
<tr>
<td>D</td>
<td>/</td>
<td>12</td>
<td>12</td>
<td>37.1±0.9</td>
<td>37.0±0.7</td>
</tr>
</tbody>
</table>

The sign “/” means the rats were injected with the same volume of normal saline instead of ZPS.

Table 2. Protective effect of puerarin to SIRS rats injected with 1000 mg/kg zymosan-A.

<table>
<thead>
<tr>
<th>Group</th>
<th>Case (n)</th>
<th>Injection</th>
<th>Survival/death (n)</th>
<th>Temperature (°C)</th>
<th>Total WBC (×10^3/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>12</td>
<td>Zymosan-A</td>
<td>2/10</td>
<td>36.9±0.96</td>
<td>35.72±1.20</td>
</tr>
<tr>
<td>B</td>
<td>12</td>
<td>Zymosan-A+ puerarin</td>
<td>10/2</td>
<td>37.13±1.05</td>
<td>36.05±1.25</td>
</tr>
<tr>
<td>C</td>
<td>12</td>
<td>Normal saline</td>
<td>0/12</td>
<td>37.20±1.12</td>
<td>37.40±0.75</td>
</tr>
</tbody>
</table>

Table 3. Alteration of TNF-α, IL-6 and IL-10 levels in puerarin-treated SIRS rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Injection</th>
<th>The concentration of cytokine</th>
<th>Temperature (°C)</th>
<th>Total WBC (×10^3/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNF-α (pg/ml)</td>
<td>IL-6 (pg/ml)</td>
<td>IL-10 (ng/ml)</td>
</tr>
<tr>
<td>A</td>
<td>Zymosan-A</td>
<td>30.87±6.81</td>
<td>525.20±92.45</td>
<td>1.37±0.17</td>
</tr>
<tr>
<td>B</td>
<td>Zymosan-A+ puerarin</td>
<td>16.71±3.75</td>
<td>399.30±77.87</td>
<td>1.95±0.17</td>
</tr>
<tr>
<td>C</td>
<td>Normal saline</td>
<td>4.50±2.03</td>
<td>105.78±43.09</td>
<td>2.21±0.21</td>
</tr>
</tbody>
</table>

 kits according to the manufacturer's instructions.

Statistical analysis

Data of cytokine content were expressed as mean ± standard deviation (x ±s) and analyzed by t test. Data of survival rate were analyzed by Chi-square test. A value of p < 0.05 was considered statistically significant.

RESULTS

Evaluation of the SIRS rat model

All rats survived after intraperitoneal injection of zymosan-A with a dose of 500 mg/kg or 750 mg/kg for 48 h, while only two rats survived after injection of 1000 mg/kg zymosan-A. However, only the manifestations (rectal temperature and the number of peripheral WBC) in rats treated with 750 or 1000 mg/kg zymosan-A met the diagnostic criteria for SIRS (Table 1).

Protective effect of puerarin to SIRS rats

About 75% (9/12) of rats treated with 1000 mg/kg zymosan-A only (Group A) died within 48 h. On the contrast, the mortality of rats injected with 62.5 mg/kg puerarin after 1000 mg/kg zymosan-A treatment (Group B) was 16.7% (2/12) (Table 2). Analysis showed significant difference in mortality between Group A and Group B (χ² = 8.22, p <0.01).

Changes of cytokine levels in SIRS rats

All rats injected with 750 mg/kg zymosan-A (Group A) or with both 750 mg/kg zymosan-A and 62.5 mg/kg puerarin (Group B) survived accompanied by significantly increased peripheral TNF-α, IL-6 and IL-10 levels. Compared to Group A, the levels of TNF-α and IL-6 of rats in Group B were markedly decreased (t = 3.62, 4.25; p <0.01) accompanied by dramatically increased IL-10 level (t = 2.95, p <0.01) (Table 3).
DISCUSSION

SIRS is frequently observed following severe infection, trauma, major operation, shock, acute severe pancreatitis and ischemia/reperfusion injury, etc. It is characterized by persistent hypermetabolism, hyperkinetic circulatory state and hyperactive inflammatory response, usually resulting in MODS and MOF with high mortality (Werdan et al., 2009; Hoesel and Ward, 2004; Netea et al., 2003; Miyaoka et al., 2005). There are numerous SIRS-associated with inflammatory factors, which are classified into pro- and anti-inflammatory cytokines, involved in the occurrence and development of SIRS (Riewald and Ruf, 2003; Ely et al., 2003). The pro-inflammatory cytokines include TNF-α, IL-1, IL-2, IL-6, IL-8, IL-12 and IFN-γ, etc., which were found to be over-expressed in SIRS rats. The anti-inflammatory cytokines consisted of IL-4, IL-5, IL-10, IL-13 and so on, which were usually noted to be low-synthesized at the early stage of SIRS. Among these cytokines, TNF-α, IL-6 and IL-10 have been considered to be the most important in the development of SIRS and MODS (Ferrer et al., 1998; Riewald and Ruf, 2003; Ely et al., 2003).

TNF-α proved to be a key factor in the initiation of inflammation cascade in SIRS and it is able to not only activate various inflammatory cells but also decrease angiogenesis and myocardial contractility as well as increase vascular permeability (Netea et al., 2003; Morris et al., 2002; Chadzinska et al., 2005). IL-6 is a potent factor being able to amplify inflammation cascade and cause tissue injury in SIRS (Miyaoka et al., 2005; Ferrer et al., 1998; Cunneen and Cartwright, 2004).

On the contrary, IL-10 is a critical inhibitor which plays an important role in suppressing the synthesis of SIRS-related pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α (Miyaoka et al., 2005; Ferrer et al., 1998). Therefore, TNF-α, IL-6 and IL-10 were selected as the target cytokines to explore the potential mechanism underlying protective effects of puerarin on experimental SIRS.

Peritoneum is the largest epithelial tissue in human and easily develops to SIRS under the stimulus of a variety of infectious or non-infectious agents (Morris et al., 2002; Chadzinska et al., 2005; Cunneen and Cartwright, 2004). Therefore, intraperitoneal injection was performed in the present study with different doses of zymosan-A to establish an experimental rat SIRS model. Our results showed that administration of 500 mg/kg zymosan-A could not cause SIRS in rats while typical manifestations of SIRS were observed in rats treated with 750 or 1000 mg/kg zymosan-A. Particularly, administration with high dose of zymosan-A (1000 mg/kg) could yield 75% mortality in the rats. These data indicated that the induction of SIRS with zymosan-A in rats was in a dose dependent manner, and the SIRS rat model can be applied to screen potential drugs for the treatment of SIRS.

A variety of drugs currently used clinically are derived from plants. As mentioned previously, puerarin, a compound extracted from roots of Puerarin thomsonii and Puerarin lobata, showed the inhibitory effects on the inflammatory responses in brain or heart ischemia/reperfusion injury, implying puerarin might be a potential drug applied in the treatment of SIRS (Yang and He, 2003; Zhu and Yao, 2001). Our results demonstrated that the mortality of rats injected with 1000 mg/kg zymosan-A and 62.5 mg/kg puerarin (16.7%) was significantly lowered compared to those injected with 1000 mg/kg zymosan-A alone (75%) (P < 0.01). The same dosage of puerarin could effectively down-regulate the peripheral levels of TNF-α and IL-6 (P < 0.01), and up-regulate that of IL-10 (P < 0.01). These findings suggested puerarin could confer therapeutic effects on experimental SIRS via regulating the expression of SIRS-related cytokines. Recently, Xu et al. (2005) and Zheng et al. (2009) reported that the neuroprotection of puerarin against cerebral ischemia was associated with the suppression of cell apoptosis by puerarin, and therapeutic effects of puerarin in non-alcoholic fatty liver rat model were related with the regulation of leptin signal transduction through JAK2/STAT3 pathways by puerarin. Therefore, further studies are needed to clarify the potential pharmacodynamic mechanism of puerarin in SIRS rats.

Limitations

So far, a majority of cytokines were reported to be involved in the pathogenesis of SIRS. Although TNF-α, IL-6 and IL-10 have been confirmed as the critical cytokines involved in the development of SIRS, protective effects of puerarin on SIRS through regulating inflammatory cytokines such as IL-1, IL-2, IL-4, IL-5, IL-8, IL-12, IL-13 and IFN-γ remain to be determined.

CONCLUSIONS

Puerarin exerted protective effects on experimental SIRS rats. Down-regulation of pro-inflammatory TNF-α and IL-6, and up-regulation of anti-inflammatory IL-10 might be involved in the protective effects of puerarin against SIRS.

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