Original Article

Cordyceps sinensis preserves intestinal mucosal barrier and may be an adjunct therapy in endotoxin-induced sepsis rat model: a pilot study

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Abstract: Background: Cordyceps sinensis (C. sinensis), a traditional Chinese medicine, exhibits various pharmacological activities such as reparative, antioxidant, and apoptosis inhibitory effects. Intestinal barrier dysfunction plays a vital role in the progression of sepsis. We aimed to explore the effect of C. sinensis on the gut barrier and evaluate its efficacy in sepsis. Methods: A murine model of gut barrier dysfunction was created by intraperitoneal injection of endotoxin. C. sinensis or saline was administered orally after the induction of sepsis. Alterations of intestinal barrier were evaluated and compared in terms of epithelial cell apoptosis, proliferation index (PI), intercellular tight junction (TJ) and proliferating cell nuclear antigen (PCNA). Results: C. sinensis significantly decreased the percentage of apoptotic cells and promoted mucosal cells proliferation indicated by enhanced PI and PCNA expression in the intestinal mucosa compared to control group. The TJs between epithelial cells which were disrupted in septic rats were also restored by treatment of C. sinensis. In survival studies, C. sinensis was demonstrated to confer a protection against the lethal effect of sepsis. Conclusion: These results suggest that C. sinensis has gut barrier-protection effect in endotoxin-induced sepsis by promoting the proliferation and inhibiting the apoptosis of intestinal mucosal cells, as well as restoring the TJs of intestinal mucosa. C. sinensis may have the potential to be a useful adjunct therapy for sepsis.

Keywords: Cordyceps sinensis, intestinal barrier, gut barrier, sepsis

Introduction

Cordyceps sinensis (C. sinensis), a parasitic fungus growing on the larvae of Lepidopetera, is a renowned traditional Chinese medicinal material. To date a variety of compounds have been extracted and purified from C. sinensis, including cordycepin and its derivatives, cordycepic acid, ergosterol, polysaccharides, nucleosides and other compounds [1]. C. sinensis has been reported to exhibit various pharmacological activities, consisting of antiaging, reparative, anti-cancer/anti-tumor, immunomodulatory, antioxidant, anti-inflammatory and apoptosis inhibitory effects [2, 4]. It is commonly used for the treatment of hyposexulaities, hyperglycemia, hyperlipidemia, asthenia, respiratory disease, renal dysfunction and renal failure, arrhythmias and other heart disease, and live disease [5].

The gut, acting as a barrier against invading pathogens, is hypothesized to play a vital role in the progression of sepsis and multiple organ dysfunction syndrome. Critical illness injures the intestinal integrity by increasing epithelial apoptosis and permeability and by decreasing epithelial proliferation and mucus integrity [6]. In sepsis, a state of severe oxidative stress is commonly encountered, which aggravates the damage of the intestinal barrier and eventually leads to bacterial translocation [7, 8]. Given the tonic effect on multiple organs and the counteraction to the above pathological process of C. sinensis, we hypothesized that C. sinensis may restore gut barrier function in injured intestinal mucosa and improve the survival of septic rats.

Therefore in the present study, we created a murine model of gut barrier dysfunction after the induction of sepsis by intraperitoneal injec-
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Our pilot study provides evidence for the clinical application of C. sinensis as a useful adjunct therapy for sepsis.

Materials and methods

Rats and materials

Wild-type healthy Sprague-Dawley (SD) male rats, weighing between 150 and 200 g, were purchased from the animal experiment center of Jinling hospital (305 East Zhongshan Road, Nanjing, 210002, P.R. China). Rats were housed in a temperature- and light-controlled room (21-25°C; 12 h light-dark cycle) for at least 2 weeks to recover from transport. All animals received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. The experimental study was approved by the animal research committee of Nanjing University (22 Hankou Road, Nanjing, 210002, P. R. China).

C. sinensis powder (Zhongmei Huadong Pharmaceutical, Hangzhou, China) was dissolved in normal saline (200 mg/ml) to prepare the turbid liquid suspension. The propidium iodide kit was purchased from Lianke Biotechnology Ltd, Shanghai, China. Endotoxin and proliferating cell nuclear antigen (PCNA) kits for immunohistochemistry were obtained from Jingmei Company, Shenzhen, China.

Experimental protocol

The rats were randomly assigned to three groups: (i) Group A, control; (ii) Group B, endotoxin-treated and (iii) Group C, endotoxin plus C. sinensis. The rats in Group B and C were administered 10 mg/kg endotoxin on the first day of experiment by intraperitoneal injection (i.p.). Each rat in Group C was additionally fed with the C. sinensis turbid liquid suspension (5 g/kg/day) daily by gavage (p.o.). The rats in Group A and B were given 0.4 ml/kg saline orally for comparison. Free access to food and water was allowed in all animals. Each group was divided into four subgroups, corresponding to 0, 4, 7 and 10 days after experimentally induced sepsis. Six animals from each subgroup were sacrificed by cervical dislocation and the intestines were harvested for detection of several markers of gut barrier, including epithelial cell apoptosis, proliferation index (PI), intercellular tight junction (TJ) and PCNA.

In survival studies, a separate group of rats was treated with C. sinensis (5 g/kg/day, p.o.) or sterile saline after induction of sepsis by intraperitoneal injection of endotoxin (20 mg/kg). Each group containing 10 rats was monitored daily for a maximum of 7 days.

Propidium iodide staining

The mucosa was scraped mechanically from 5 cm of the intestine, and then a single-cell suspension was prepared with the Medimachine system (BD Biosciences, San Jose, CA). Freshly isolated mucosal cells were washed twice in PBS, resuspended a hypotonic fluorochrome solution [50 μg/ml propidium iodide (Sigma) in 0.1% sodium citrate plus 0.1% Triton X-100], and incubated for 2 h, allowing the penetration of propidium iodide into the nucleus and binding within the DNA breaks. Cells were then analyzed by flow cytometry using the FACSCalibur. The percentage of apoptotic cells was detected after proper gating on DNA content as described in previous studies [9]. The proportion of cells in S+G2/M phase represents the PI of the mucosa or the synthetic rate of DNA.

Intercellular tight junction evaluation

The intestine was cut into 1 mm × 1 mm × 2 mm samples and immediately pre-fixed with 3% glutaraldehyde-1.5% paraformaldehyde-0.1 mol/L phosphate buffer (pH 7.2) at 4°C for 2 hours. Then, the samples were washed and post-fixed with 1% osmium tetroxide at 4°C for 1.5 hours. After gradient dehydration with alcohol and acetone, the samples were embedded in epoxy resin 618 and then cut into ultrathin sections (60 nm). The slides were stained with uranyl acetate and lead citrate, and then photographed using transmission electron microscope (JEOL-100 CX) at 80 kV.

PCNA staining

Briefly, the intestines were fixed in formalin and embedded in paraffin. 6 μm sections cut from
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Figure 1. Representative studies of cell cycle phase using flow cytometry. Proliferation index (PI) denotes the proportion of cells in S+G2/M phase. A-D (Group B, endotoxin-treated) and E-H (Group C, endotoxin plus C. sinensis) correspond to 0, 4, 7 and 10 days after endotoxin-induced sepsis respectively.
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paraffin blocks were dewaxed and rehydrated in graded concentrations of ethanol to distilled water. Following deactivation of the endogenous peroxidases with 3% (v/v) H$_2$O$_2$ for 10 minutes, antigen retrieval was then performed in 0.01 M citrate buffer (pH 6.0). After incubation with a PCNA monoclonal antibody (Jingmei, China) at 37°C for 40 minutes, a horseradish peroxidase-conjugated second antibody at 37°C for 20 minutes was used. The slides were observed by a microscope after treated with diaminobenzidine (DAB) and counterstained with hematoxylin. Well-formed crypts with longitudinal profiles were selected for evaluation.

Table 1. Apoptosis and PI of intestinal mucosal cells of rats in three groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0 Apoptosis</th>
<th>Day 0 PI</th>
<th>Day 4 Apoptosis</th>
<th>Day 4 PI</th>
<th>Day 7 Apoptosis</th>
<th>Day 7 PI</th>
<th>Day 10 Apoptosis</th>
<th>Day 10 PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.66±1.96</td>
<td>9.88±0.49</td>
<td>4.81±1.81</td>
<td>9.57±0.68</td>
<td>4.70±1.95</td>
<td>9.78±0.63</td>
<td>4.86±1.86</td>
<td>9.82±0.47</td>
</tr>
<tr>
<td>B</td>
<td>4.70±1.94</td>
<td>9.67±0.53</td>
<td>32.84±6.42</td>
<td>7.22±0.50</td>
<td>16.46±5.58</td>
<td>8.03±0.39</td>
<td>9.52±2.98</td>
<td>9.04±0.42</td>
</tr>
<tr>
<td>C</td>
<td>4.82±1.92</td>
<td>9.79±0.51</td>
<td>19.10±5.94</td>
<td>8.21±0.51</td>
<td>4.64±2.06</td>
<td>2.28±1.02</td>
<td>10.28±0.32</td>
<td>10.28±0.32</td>
</tr>
</tbody>
</table>

Group A: control; Group B: endotoxin-treated; Group C: endotoxin plus C. sinensis (6 rats per subgroup). a. compared with Group A, P<0.05; b. compared with Group B, P<0.05.

Figure 2. Tight junction structural morphology in the intestinal mucosa. A-D (Group B, endotoxin-treated) and E-H (Group C, endotoxin plus C. sinensis) correspond to 0, 4, 7 and 10 days after endotoxin-induced sepsis respectively (transmission electron microscopy, × 26 500).

The immunohistochemical characteristics from the bottom of the crypt up to the border of the villus-crypt junction were reviewed at a magnification of ×400. The positive staining for PCNA was orange or brown in the cytoplasm and/or nucleus. For each sample, at least 10 crypts were reviewed to calculate the proportion of positive cells.

Statistical analysis

Data were presented as mean ± standard deviation (SD). One-way ANOVA was used for comparison among three groups. The Student-
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Newman-Keuls (SNK) test was applied for post-hoc comparisons, when necessary. The cumulative survival rate was manifested by the Kaplan-Meier method. All analyses were performed using IBM SPSS for Windows version 19.0 and P < 0.05 was considered significant.

Results

Apoptosis and PI of intestinal mucosal cells

We identified apoptotic mucosal cells by flow cytometry with the propidium iodide staining method (Figure 1). Compared with control group, a consistently high level of apoptotic cells was noticed in Group B after intraperitoneal injection of endotoxin (Table 1). The percentages of apoptotic cells in Group B reached 32.84±6.42%, 16.46±5.58% and 9.52±2.98 at day 4, 7 and 10 respectively. Treatment with C. sinensis significantly decreased the percentage of apoptotic cells in the murine model of gut barrier dysfunction. The remarkable apoptosis inhibitory effect of C. sinensis even resulted in the less proportion of apoptotic mucosal cells compared with control group at day 10 (2.28±1.02% vs. 4.86±1.86%). As for PI of intestinal mucosal cells, a significant decrease was observed in the endotoxin-induced sepsis rats at day 4 and day 7 compared to control group. The PI in group C achieving 10.28±0.32% at day 10 was higher than that of control group (9.82±0.47%), which suggested the promotion of mucosal cells proliferation by C. sinensis.

Intercellular tight junctions of intestinal mucosa

Ultrastructural morphology of the intestinal mucosa was shown in Figure 2. Before treatment with endotoxin, regularly aligned microvilli and intact TJs were observed in the intestinal epithelium (Figure 2A, 2E). There were TJs and villi disruption, loose microvilli, and swollen organelles with decreased electron density in both the Group B and C at day 4 (Figure 2B, 2F). The TJs between epithelial cells were broadened remarkably at day 4 in Group B (442.54±17.28 µm) and Group C (409.03±16.82 µm) in contrast with the control group (313.53±20.03 µm) as indicated in Table 2. At day 7 and day 10 postoperatively, the microvilli in the intestinal epithelium restored and the TJs were gradually integrated and distinct (Figure 2C, 2D, 2G, 2H). Compared to the endotoxin-treated group, the intestinal mucosa of the rats receiving endotoxin plus C. sinensis manifested a significantly tighter intercellular space at day 7 (260.94±14.76 µm vs. 389.16±18.15 µm) and day 10 (229.47±15.89 µm vs. 337.57±16.40 µm).

PCNA expression in the intestinal mucosa

PCNA was used as an indication of cellular proliferation. PCNA-positive cells were moderately detected in the basal parts of the intestinal mucosa before treatment (Figure 3A, 3E). That may be attributed to the self-renew of epithelial cells deriving from the intestinal stem cells located in the mucosa crypts. The number of PCNA-positive mucosal cells decreased significantly at day 4 after treatment (Figure 3B, 3F) with 12.04±3.28% in Group B and 12.92±4.20% in Group C compared to 19.02±3.14% in control group (Table 3). There was a rise in the proportion of PCNA-positive cells in the intestinal mucosa of Group B at day 7 and 10 (Figure 3C, 3D; Table 3). Rats in the C. sinensis-treated group showed a stronger expression of PCNA in the intestinal mucosa than those injected with endotoxin only at day 7 and 10 (Figure 3G, 3H; Table 3). The percentage of PCNA-positive cells was 24.93±3.41% and 30.46±5.24% in Group C at day 7 and 10, significantly higher than that in Group B (19.16±3.25% and 19.57±4.40% respectively).

Survival of septic rats

To further determine the effect of C. sinensis systemically, we evaluated the survival rates of rats injected intraperitoneally with endotoxin (20 mg/kg) and subsequently treated orally with saline or C. sinensis (Figure 4). 30% of sep-

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**Table 2. Intercellular tight junction of intestinal mucosa of rats in three groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>336.81±17.46</td>
<td>313.53±20.03</td>
<td>322.38±16.26</td>
<td>321.68±18.73</td>
</tr>
<tr>
<td>B</td>
<td>326.52±19.92</td>
<td>442.54±17.28</td>
<td>389.16±18.15</td>
<td>337.57±16.40</td>
</tr>
<tr>
<td>C</td>
<td>343.64±15.74</td>
<td>409.03±16.82</td>
<td>260.94±14.76</td>
<td>229.47±15.89</td>
</tr>
</tbody>
</table>

Group A: control; Group B: endotoxin-treated; Group C: endotoxin plus C. sinensis (6 rats per subgroup). a. compared with Group A, P<0.05; b. compared with Group B, P<0.05.
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This pilot study was conducted to investigate the effect of C. sinensis on the gut barrier function in the rats with endotoxin-induced sepsis. Injured intestinal mucosa characterized by TJ fracture, ultrastructure damage and increased apoptosis of epithelial cells in septic rats, was ameliorated by in vivo administration of C. sinensis. The survival of rats injected by endotoxin to partly imitate human sepsis was also shown to increase by treatment of C. sinensis. Our results may provide evidence for the clinical application of C. sinensis as an adjunct therapy for sepsis.

C. sinensis is a well-known endoparasitic fungus which has been used as a tonic and medicinal food in traditional Chinese medicine for centuries. A large body of in vitro and vivo studies including open-label and double-blinded clinical trials have been conducted, showing intriguing results that support its beneficial role in various illnesses [10]. C. sinensis also possesses the merits of safety and nontoxicity and in most cases the median lethal dose of C. sinensis and its mycelial fermentation products could not even be detected. In toxicity studies [11, 12], no deaths of mice were observed even when given high oral dosage of C. sinensis up to 80 g/kg.

So far there is insufficient data regarding the effects that C. sinensis exerts on the intestinal system. Koh et al. [13] found that C. sinensis may enhance the secretion of granulocyte-macrophage colony stimulating factor (GM-CSF)

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18.82±3.56</td>
<td>19.02±3.14</td>
<td>19.58±3.53</td>
<td>19.77±3.23</td>
</tr>
<tr>
<td>B</td>
<td>17.97±3.85</td>
<td>12.04±3.28</td>
<td>19.16±3.25</td>
<td>19.57±4.40</td>
</tr>
<tr>
<td>C</td>
<td>18.67±3.24</td>
<td>12.92±4.20</td>
<td>24.93±3.41</td>
<td>30.46±5.24</td>
</tr>
</tbody>
</table>

Group A: control; Group B: endotoxin-treated; Group C: endotoxin plus C. sinensis (6 rats per subgroup). a. compared with Group A, P<0.05; b. compared with Group B, P<0.05.
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from Peyer’s patch cells of C3H/HeJ mice fed with C. sinensis. Notably, GM-CSF, a well-characterized specific glycoprotein participating in the regulation of production, differentiation and function of the granulocytes and monocyte-macrophages, has been proved to enhance survival of sepsis related to an improvement in gut barrier function [14]. Another study using radiation-induced intestinal injury model demonstrated that C. sinensis reduced the number of apoptotic cells in both the crypts and lamina propria in parallel with an increased number of surviving crypt stem cells [15]. Unsurprisingly, C. sinensis was found to inhibit the apoptosis of intestinal mucosal cells as well in our experiment. However an in vitro study [16] of intestinal epithelial cell HT29 showed indomethacin-induced apoptosis was not affected by C. sinensis. Despite that, C. sinensis indeed stimulated HT29 cell proliferation remarkably, which was inspiring and corroborated the potential benefit of C. sinensis in gut barrier. Moreover, C. sinensis also significantly reduced the amount of gastric injury in the rats receiving indomethacin orally.

Antioxidant activity of natural and cultured C. sinensis has been proved conclusively in vitro and vivo studies [17-20]. Reactive oxygen species (ROS) are a group of chemicals including several oxygen metabolites such as superoxide anion, hydroxyl radical and hydrogen peroxide that have been implicated in the pathophysiology of various chronic illness and aging [21]. Excessive ROS damage cellular proteins involving cytoskeletal proteins and, ultimately, disrupt gut barrier to increase intestinal permeability [22]. It is therefore reasonable to infer antioxidant effect as one possible mechanism through which C. sinensis helps preserve the intestinal barrier.

Matrix metalloproteases (MMPs) can mediate degradation of TJ proteins including occluding, ZO-1, and claudins, causing damage of barrier function in epithelial and endothelial cells [23-25]. MMPs inhibitors were demonstrated to restore the loss of TJ proteins and increased permeability of intestinal epithelial Caco-2 cell monolayers [26]. Previous studies have suggested that C. sinensis down-regulated MMPs in liver and lung tissues as well as melanoma cells [27-30]. C. militaris, another Cordyceps species, decreased the level of MMP-3 and MMP-9 in the intestinal tissue of mice with DSS-induced colitis [31]. Unlike C. sinensis whose stromata have not been artificially cultivated and having a limited distribution, C. militaris distributes worldwide and its stromata can be easily cultivated. It has been increasingly studied as a substitute for C. sinensis because of their very similar chemical capacities and medicinal properties [32]. Therefore we here take its actions on the intestine as a reference of C. sinensis and surmise that the restoration of TJs in the gut by C. sinensis may in part result from MMPs inhibition.

C. sinensis increased the survival of rats after induction of sepsis by endotoxin. In addition to the preservation of gut barrier which plays a pivotal role in the progression of sepsis [6], the wide spectrum of pharmacological actions in renal, hepatic and respiratory systems as well as immunomodulatory effects [33] of C. sinensis may contribute collectively to the improved outcomes. In this pilot study, we explored the benefits of C. sinensis powder, the most common form seen commercially. The biologically active constituents of C. sinensis including polysaccharides, nucleosides and sterols and their structures have already been well-established at present [33]. The endotoxin-injected
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rat model that we used has been extensively described and resembles human sepsis. However, given the multi-hit property and complexity of human sepsis, the efficacy of C. sinensis on sepsis requires to be evaluated in clinical trials.

In conclusion, our results indicate that C. sinensis can improve the dysfunction of gut barrier in septic rats by promoting the proliferation and inhibiting the apoptosis of intestinal mucosal cells, as well as restoring the TJs of intestinal mucosa. C. sinensis showed curative effects in improving the survival of septic rats and may be a useful adjunct therapy for sepsis. However, further studies are warranted to better characterize the ingredients of C. sinensis responsible for the gut barrier-protection effect and to confirm the efficacy in human sepsis.

Acknowledgements

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Disclosure of conflict of interest

None.

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