Original Article

Impact of clostridium difficile infection on immune function in patients with ulcerative colitis and the clinical nursing observation

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Abstract: Objective: To investigate the impact of Clostridium difficile infection (CDI) on immune function in patients with ulcerative colitis and the clinical nursing effect. Methods: A total of 93 active patients with ulcerative colitis from January 2015 to December 2017 in our hospital were selected and randomly divided into two groups, the control group (n=71) and the infection group (n=22), according to whether the patients were infected by Clostridium difficile (C. diff). The baseline characteristics including age, gender, and degree of active phase were gathered and put into comparisons. Enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP), procalcitonin (PCT) and high mobility group box protein 1 (HMGB1) in sera of all patients and the C. diff toxins in stool samples of the patients. And western blotting was used to detect the expression levels of Toll-like receptor 4 (TLR4) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in peripheral blood lymphocytes of all patients. Results: A total of 22 cases with C. diff toxins out of 93 cases with ulcerative colitis were detected, and the detection rate was 23.66%. The levels of IL-1β, IL-6, IL-8, TNF-α, CRP, PCT and HMGB1 in serum of the infection group were higher than these of the control group. The expression levels of TLR4 and NF-κB in peripheral blood lymphocytes of the infection group were significantly higher than those of the control group. Conclusion: CDI can lead to immune dysfunction in patients with ulcerative colitis, which may be related to activation of HMGB1/TLR4/NF-κB signaling pathway, suggesting that HMGB1 detection can be performed in clinical care for early diagnosis of CDI in patients with ulcerative colitis.

Keywords: Clostridium difficile, ulcerative colitis, immune function, high mobility group box-1 protein, nursing observation

Introduction

Ulcerative colitis is a chronic recurrent inflammatory bowel disease with alternating remission and active phase, in which inflammation and ulceration of the intestinal mucosa are main pathological manifestations. As for this condition, clinical treatments like administration of antibiotics, glucocorticoids and immunomodulators or surgery are often applied [1]. However, long-term application of drugs such as antibiotics can lead to alteration of intestinal microflora and immune disorder, which increases the risk of CDI in patients with ulcerative colitis. According to the literature, stool specimens in about 5% to 40% of patients with ulcerative colitis are tested positive for C. diff [2, 3]. In addition, CDI can lead to poor prognosis in patients with ulcerative colitis [4, 5]. Murthy and other scholars found that the 5-year mortality rate in patients with both ulcerative colitis and CDI was 27%, while the 5-year mortality rate in patients with only ulcerative colitis was 14% [6]. Hence, investigation of the impact of Clostridium difficile infection on immune function in patients with ulcerative colitis is of great significance to targeted nursing monitoring and clinical observation in the future.

High mobility group box protein 1 (HMGB1) is a highly-conserved non-histone nucleoprotein that can be released in the interstitial fluid and blood circulation when the histocytes are stimulated by external stimuli such as infection,
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wounds, and burns. Then HMGB1 involved in the chemotaxis and activation of lymphocytes through the Toll-like receptor 4 (TLR4)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway induces the lymphocytes to secrete IL-1β, IL-6, IL-8, TNF-α and other inflammatory cytokines, which further aggravates topical and systemic inflammatory response of the tissues [7, 8]. Previous studies found that the expression level of HMGB1 was closely related to the severity of patients with ulcerative colitis and could be regarded as a reliable indicator of reflecting intestinal inflammation [9]. Nevertheless, the role of HMGB1 and its mediated TLR4/NF-κB signaling pathway in CDI among patients with ulcerative colitis is unclear. Therefore, 93 patients with active ulcerative colitis were selected and divided into the control group and the infection group according to whether the patients were infected by C. diff. The differential expressions of HMGB1, TLR4, NF-κB as well as IL-1β and other inflammatory cytokines were compared between the two groups, and the effect of CDI on immune function in patients with ulcerative colitis was investigated from the perspective of HMGB1/TLR4/NF-κB signaling pathway.

Materials and methods

Patient selection

A total of 93 active patients with ulcerative colitis admitted to The Second Affiliated Hospital of Nanchang University from January 2015 to December 2017 were selected and divided into the control group (n=71) and the infection group (n=22) according to whether the patients were infected by C. diff. The differential expressions of HMGB1, TLR4, NF-κB as well as IL-1β and other inflammatory cytokines were compared between the two groups, and the effect of CDI on immune function in patients with ulcerative colitis was investigated from the perspective of HMGB1/TLR4/NF-κB signaling pathway.

Degree of active phase

The severity of active phase in the patients were graded according to the modified Truelove and Witts' severity index. In a mild active phase, the number of bowel movement was fewer than 4 times per day; there was no or a small amount of blood in the stools; pulse, body temperature and hemoglobin level were normal; and erythrocyte sedimentation rate was below 20 mm per hour. In a severe active phase, the number of bowel movement was 6 times per day or more; blood in the stools was visible; pulse rate was above 90 bpm; body temperature was greater than 37.8°C; hemoglobin level was below 75% of normal value; and the erythrocyte sedimentation rate was greater than 30 mm per hour. The condition between mild and severe in severity was considered as a moderate one.

Enzyme linked immunosorbent assay (ELISA)

ELISA was used to detect the levels of interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP), procalcitonin (PCT) and high mobility group box protein 1 (HMGB1) in sera of all patients and the C. diff toxins in stool samples of the patients. Detailed steps were as follows: 100 mg of solid stools were extracted from a crude stool sample and put into a centrifuge tube, and were shaken into a mixture after adding 1 mL of PBS. Then the mixture was centrifuged at 3,000 rpm for 5 min, and the supernatant of the centrifuged mixture was obtained for ELISA detection (Korunda, Shenzhen, EIA4448) of C. diff toxin; 3 mL venous blood was drawn and naturally coagulated at
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**Table 1. Comparison of baseline characteristics**

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>Control group</th>
<th>Infection group</th>
<th>Statistics</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases (n)</td>
<td>71</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (year)</td>
<td>50.46±11.50</td>
<td>52.03±12.69</td>
<td>t=0.546</td>
<td>0.586</td>
</tr>
<tr>
<td>Gender (male/female, n)</td>
<td>39/32</td>
<td>13/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degree of active phase (n)</td>
<td>Mild</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Comparison of serum inflammatory cytokines between the two groups**

<table>
<thead>
<tr>
<th>Inflammatory cytokines</th>
<th>Control group</th>
<th>Infection group</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β (ng/L)</td>
<td>84.36±21.05</td>
<td>117.01±28.64</td>
<td>5.812</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 (ng/L)</td>
<td>34.45±8.92</td>
<td>97.61±17.39</td>
<td>22.620</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-8 (ng/L)</td>
<td>30.17±13.45</td>
<td>37.70±15.81</td>
<td>2.200</td>
<td>0.030</td>
</tr>
<tr>
<td>TNF-α (ng/L)</td>
<td>25.09±8.51</td>
<td>48.96±11.44</td>
<td>10.550</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>11.26±3.98</td>
<td>88.49±18.90</td>
<td>32.540</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PCT (μg/L)</td>
<td>0.22±0.13</td>
<td>1.19±0.26</td>
<td>23.510</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: IL-1β, interleukin 1β; IL-6, interleukin 6; IL-8, interleukin 8; TNF-α, tumor necrosis factor-α; CRP, C-reactive protein; PCT, procalcitonin.

The expression levels of TLR4 and NF-κB in peripheral blood lymphocytes were detected using WB. The specific steps were as follows: 5 mL of venous blood was pipetted out to the anticoagulant tube, and mixed with 5 mL of normal saline. Then the mixture was added to the surface of lymphocyte separation medium (10 mL) (Huayueyang, Beijing, GH5003), followed by centrifugation at 400 g for 20 min. After being centrifuged, the solution appeared 4 layers visibly. The first layer was the plasma layer; the second was the milky lymphocyte; the third was the transparent separation medium; the fourth was the red blood cell. The lymphocytes in the second layer were aspirated into a syringe and then injected into a centrifuge tube containing 5 mL of normal saline; the solution was centrifuged at 400 g for 20 min. After 3 washes, peripheral blood lymphocytes were obtained. A certain amount of cells (1 * 10⁶) were taken and added into 100 μL of RIPA lysate (Beyotime, Shanghai, P0013) for full lysis. Then the supernatant was obtained for BCA protein quantification (Beyotime, Shanghai, P0012). A small amount of total proteins (40 μg) were subjected to polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. Subsequent to the transferring, 5% of bovine serum albumin was applied to membrane sealing at room temperature for 2 h. Then the membrane was incubated overnight at 4°C using anti-TLR4 antibody (1:500, Abcam, Shanghai, ab13556), anti-NF-κB antibody (1:1,000, Abcam, Shanghai, ab32360) and anti-GAPDH antibody (1:2,500, Abcam, Shanghai, ab9485). After three washes for the overnight membrane, horseradish peroxidase-labeled goat anti-rabbit secondary antibody (Amyjet, Wuhan, China 6401-05) was added into the total proteins of the membrane for incubation of 1 h. Then ECL substrate (Beyotime, Shanghai, P0018) was used for coloration; a gel imager (Bio-Rad, GelDoc 2000) was used for exposure and photographing following three washes again for the membrane. Referring to the WB quantitative method Meng et al. Mentioned, the ratio of the gray value of the target protein to GAPDH in the control group was taken as 1 [11].

**Statistical analysis**

Statistical analysis was performed using SPSS package for Windows, version 19.0. All measurement data were in a normal distribution. Measurement data in both groups are expressed as mean ± standard deviation and pro-
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Baseline characteristics

A total of 22 patients with C. diff toxins of 93 cases with ulcerative colitis were detected, and the detection rate was 23.66%. The results in Table 1 show that there was no significant difference in age, gender, and degree of active phase between the two groups (all $P>0.05$), which demonstrates the comparability of the study.

Serum inflammatory cytokines

The levels of IL-1β, IL-6, IL-8, TNF-α, CRP and PCT (all $P<0.05$) in sera of the infection group were higher than those of the control group (Table 2, Figure 1).

HMGB1 in sera

Figure 2 shows that the level of HMGB1 in sera of the infection group (4.90±0.77 μg/L) were higher than that of the control group (3.59±0.58 μg/L) ($P<0.001$).

The expression levels of TLR4 and NF-κB in peripheral blood lymphocytes

The expression levels of TLR4 and NF-κB in peripheral blood lymphocytes of the infection group were significantly higher than those of the control group (both $P<0.001$) (Figure 3).

Discussion

In recent years, the epidemiology, pathological mechanism and treatment of CDI have made great progress, but the infection still remains [12-14]. It is a major risk factor for poor prognosis in patients with ulcerative colitis, which can lead to aggravation of clinical symptoms, pro-
longed hospital stay, and significant increase in long-term mortality [15, 16]. Therefore, the in-depth exploration of CDI in patients with ulcerative colitis has important clinical value. In this study, the detection rate of C. diff toxins in stool samples was 23.66%. CDI rate reported recently in patients with ulcerative colitis ranged from 5% to 40% [17]. For instance, Gu et al. found 6 cases of CDI in 84 patients with ulcerative colitis, and the infection rate was 7.1% [2]; while Kaneko et al. reported that the rate of CDI was up to 40.1% [3]. The difference might be related to some factors such as inclusion criteria, sample size or detection method of CDI. Though the cell culture cytotoxicity neutralization assay is recognized as the gold standard for CDI, it is difficult to meet the needs of clinical testing due to high experimental condition, complicated and time-consuming operation, and high cost. So, it is no longer used in most research reports [18]; and in recent years, ELISA has become the main testing method for CDI. Eastwood et al. reported the specificity of ELISA detection could reach 99.4%, which proved the availability of alternative method [19]. Therefore, ELISA was used in this study as a method for confirming the diagnosis of CDI.

HMGB1, the first one identified in the HMGB family, can get an evolutionarily highly-conserved DNA-binding protein involved in regulation of chromosome structure and repair of DNA damage in the nucleus. When cells are damaged by the outside, HMGB1 can be transferred to the cytoplasm from the nucleus and then be secreted outside the cell to participate in the regulation of immune responses such as inflammation [21]. Studies have found that intestinal inflammation and mucosal damage caused by C. diff are closely related to HMGB1-mediated inflammatory response. For instance, Liu et al. found that HMGB1 was released in large quantities when CT26 cells and mouse colon tissues were stimulated by C. diff toxins; and then the HMGB1 could further lead to increase in the levels of inflammatory cytokines such as IL-1β, IL-6 and TNF-α; blocking the expression of HMGB1 could inhibit the inflammatory response [22]. This study also found that the level of serum HMGB1 in the infection group was significantly higher than that of the control group, further confirming that the high inflammatory state of patients with ulcerative colitis caused by CDI was closely related to high expression of HMGB1. A large number of stud-
ies have confirmed that the expansion of HMGB1-mediated inflammatory effect is mainly dependent on the activation of TLR4/NF-κB signaling pathway [23]. Meng et al. found that HMGB1 mediated inflammatory response through activation of the TLR4/NF-κB signaling pathway in a lipopolysaccharide-induced inflammatory model, which could prove the dependence [11]. The results in this study also show that the expression levels of TLR4 and NF-κB in peripheral blood lymphocytes of patients with CDI were significantly higher than those of the control group, indicating that activation of the TLR4/NF-κB signaling pathway was involved in immune dysfunction caused by CDI in patients with ulcerative colitis.

Although this study initially explored the impact of CDI on immune function in patients with ulcerative colitis, there are still some shortcomings: (1) only 22 cases with ulcerative colitis was infected by C. diff in this study, and the sample size was small. So, more cases still need to be included for the validation of relevant conclusions; (2) the results in this study show that CDI could increase the expression of serum HMGB1 in patients with ulcerative colitis. But whether there is a positive correlation between the degree of inflammation caused by infection and the expression level of HMGB1 remains unclear; (3) the relation between CDI and activation of HMGB1/TLR4/NF-κB signaling pathway lacks direct and valid evidence, which requires specific inhibition of related molecules for verification through in vitro cell experiments.

In conclusion, CDI can lead to immune dysfunction in patients with ulcerative colitis, which may be related to activation of HMGB1/TLR4/NF-κB signaling pathway. It is suggested that HMGB1 detection can be performed in clinical care to early detect C. diff in patients with ulcerative colitis, which provides a basis for further clinical diagnosis and treatment.

Disclosure of conflict of interest

None.

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