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• *BRIEF REPORTS* •

# Effects of garlicin on apoptosis in rat model of colitis

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## Abstract

**AIM:** To investigate the effects of garlicin on apoptosis and expression of bcl-2 and bax in lymphocytes in rat model of ulcerative colitis (UC).

**METHODS:** Healthy adult Sprague-Dawley rats of both sexes, weighing 180±30 g, were employed in the present study. The rat model of UC was induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) enema. The experimental animals were randomly divided into garlicin treatment group (including high and low concentration), model control group, and normal control group. Rats in garlicin treatment group and model control group received intracolic garlicin daily at doses of 10.0 and 30.0 mg/kg and equal amount of saline respectively 24 h after colitis model was induced by alcohol and TNBS co-enema. Rats in normal control group received neither alcohol nor only TNBS but only saline enema in this study. On the 28th d of the experiment, rats were executed, the expression of bcl-2 and bax protein was determined immunohistochemically and the apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) method. At the same time, the rat colon mucosal damage index (CMDI) was calculated.

**RESULTS:** In garlicin treatment group, the positive expression of bcl-2 in lymphocytes decreased and the number of apoptotic cells was more than that in model control group, CMDI was lower than that in model control group. The positive expression of bax in lymphocytes had no significant difference.

**CONCLUSION:** Garlicin can protect colonic mucosa against damage in rat model of UC induced by TNBS enema.



**Key words:** Garlicin; Ulcerative colitis; Apoptosis; Bcl-2

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## INTRODUCTION

Ulcerative colitis (UC), one of the chronic nonspecific inflammation diseases of the intestine, is related to infection, heredity, and immunologic inadequacy. In recent years, studies have shown that apoptosis plays an important role in the pathogenesis of UC[1-3]. Apoptosis of enterocytes and lymphocytes are the main reasons for UC and inflammation[4,5]. Apoptosis of lymphocytes is closely related with UC[6-8]. Garlicin is a kind of chemical compound extracted from garlic corn. It has many biological activities against inflammation, fungi, anti-oxidant, and tumor, *etc.*[9-11]. Therefore, we performed this study to observe the effects of garlicin on expression of bcl-2 and bax protein in rats with UC and its possible mechanism against the damage to colonic mucosa.

## MATERIALS AND METHODS

### *Materials*

Healthy adult Sprague-Dawley rats of both sexes, weighing 180±30 g, employed in this study were purchased from the Experimental Animal Center, Hubei Academy of Medical Sciences, housed in a temperature conditioned room (22-24 ℃) with a 12-h light–dark cycle, allowed free access to standard rat chow and water *ad libitum*, and acclimatized to the surroundings for 1 wk before the experiment. The study protocol was in accordance with the guideline for animal research and approved by the Ethical and Research Committee of the hospital.

### *Reagents*

Garlicin was purchased from Hubei Wusan Drug Manufactory. 2,4,6-Trinitrobenzene sulfonic acid (TNBS) was bought from Sigma Corp. Immunohistochemical assay kits for bcl-2 and bax were provided by Wuhan Boster Reagent Corp. Apoptosis assay kits and S-P assay kits were provided by Roche Reagent Corp.

### *Experimental protocol*

Rat model of UC was induced by TNBS enema as previously described in the literature[12]. According to different treatment regimens, the experimental animals were randomly divided into garlicin treatment group (including high and low concentration), model control group, and normal control group. Rats in garlicin treatment group and model control group received intracolic garlicin daily at doses of 10.0 and

30.0 mg/kg and equal amount of saline (8:00 am) 24 h after colitis model was induced by 50% alcohol and TNBS (150 mg/kg) co-enema. Rats in normal control group received neither alcohol nor TNBS but saline enema. On the 28th d of the experiment, rats were executed, the expression of bcl-2 and bax in lymphocytes was determined immunohistochemically and apoptotic cells were detected by the TUNEL method. At the same time, the colon mucosal damage index (CMDI) was evaluated.

### Colon mucosal damage index

CMDI was evaluated by the methods as previously reported[13]. The evaluation standard of CMDI includes adhesion, congestion, ulcer inflammation, and pathological change depth. Adhesion and congestion according to the difference of pathological change degree were scored as 0, 1 and 2 respectively. The inflammation and ulcer of colonic mucosa scored 1; the pathological change of submucous membrane, muscular layer, serous coat layer scored 1, 2, and 3 separately. Each point was added to get the total points.

### Immunohistochemistry detection

The expression of bcl-2 and bax protein in colon tissue was determined immunohistochemically as previously described[14,15], formalin-fixed, paraffin-embedded tissue blocks were cut into 5-μm-thick sections mounted on glass slides, and then kept in an oven at 4 ℃ overnight. Immunostaining was performed as previously described with a slight modification[16]. Sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 min. To improve the quality of staining, microwave oven-based antigen retrieval was performed. Slides were probed with either anti-bcl-2 (1:100, mouse mAb) or anti-bax (1:100, mouse mAb). Sections were washed thrice with PBS for 10 min each and incubated with biotin-labeled anti-mouse IgG for 1 h at room temperature. After washing thrice with PBS for 10 min each, sections were stained with a streptavidin-peroxidase detection system. Incubation with PBS instead of the primary antibody served as a negative control. In specimens containing positive cells, the positive cells were counted in 10 randomly selected fields under 200- or 400-fold magnification for each sample, and the average was expressed as the density of positive cells.

### Determination of apoptosis

The TUNEL assay, originally described by Gavrieli *et al*[17], was used with minor modifications. Briefly, 5-μm-thick tissue sections were mounted onto glass slides, deparaffinized, hydrated, and treated with proteinase-K (Roche Corp.; 20 μg/mL in 10 mmol/L Tris-HCl buffer, pH 7.4) for 15-30 min at 37 ℃. Slides were rinsed twice with PBS. Then, 50 μL of TUNEL reaction mixture (450 μL nucleotide mixture containing fluoresceinated dUTP in reaction buffer plus 50 μL enzyme TdT from calf thymus, Roche Corp.) was added to the samples. To ensure homogeneous spread of the TUNEL reaction mixture on tissue sections and to avoid evaporative loss, slides were covered with coverslips during incubation and incubated in a humidified chamber for 60 min at 37 ℃. After rinsing, slides were incubated with anti-fluorescein antibody, Fab fragment from sheep, conjugated with horse-radish peroxidase for 30 min at 37 ℃. Slides were rinsed twice with PBS. Then, 50-100 μL of DAB substrate was added and incubated for 10 min at room temperature. Samples were counterstained prior to analysis by light microscopy. Positive signals were defined as presence of a distinct brown nuclear staining of the neoplastic cells or were morphologically defined as apoptotic bodies. The apoptotic index (AI) was determined by counting at least 1 000 neoplastic nuclei in 10 randomly chosen fields at 400-fold magnification. Apoptotic cells were identified by TUNEL assay in conjunction with characteristic morphological changes such as cell shrinkage, membrane blebbing, and chromatin condensation, to distinguish apoptotic cells and bodies from necrotic cells.

### Statistical analysis

All statistical analyses were performed with SPSS10.0 statistical package for Microsoft Windows. Measurement data were expressed as mean±SD, *t* test and one-way analysis of variance were used to compare continuous variables among groups. $P<0.05$ was considered statistically significant.

## RESULTS

### Protective effects of garlicin on rat colonic lesion

Pronounced pathological changes of colonic mucosa were similar to those in human IBD which were observed in rats colitis model induced by both alcohol and TNBS co-enema. CMDI significantly increased in experimental animals compared to normal controls ($P<0.01$). Treatment groups with different doses of garlicin could effectively reduce the severity of gut injury and CMDI significantly decreased in a dose-dependent manner in rats treated with garlicin compared to that in model control group ($P<0.05$-$0.01$, Table 1).

### Effects of garlicin on expression of bcl-2 and bax protein

In the present study, the expression of bcl-2 and bax was

**Table 1** Effect of garlicin on CMDI in rats with experimental colitis (mean±SD, $n = 10$)

| Group | CMDI | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Normal control | 2 | 5 | 3 | | | | | |
| Model control | | | | 1[d] | 2[d] | 3[d] | 3[d] | 1[d] |
| Garlicin (10.0 mg/kg) | | 2[a] | 2[a] | 3[a] | 2[a] | 1[a] | | |
| Garlicin (30.0 mg/kg) | | | 5[b] | 3[b] | 2[b] | | | |

[a]$P<0.05$, [b]$P<0.01$ *vs* model control; [d]$P<0.01$ *vs* normal control.

also investigated by immunohistochemistry. Immunostaining specific for bcl-2 and bax was shown as brown. Positive expression of bcl-2 in lymphocytes significantly increased in rat model of UC induced by alcohol and TNBS enema compared to the normal control group ($P<0.01$), which were significantly inhibited by different doses of garlicin ($P<0.01$-0.05 *vs* model control, Table 2 and Figure 1). Positive expression of bax in lymphocytes was common in normal group, which had no alteration in model control group and garlicin treatment group.

### *Apoptosis*

TUNEL staining was restricted to the nuclei of apoptotic cells. TUNEL-positive staining cells were detected in normal control group, model control group, and garlicin treatment group. The AI significantly decreased ($P<0.01$) in model control group compared to normal control group, and increased in garlicin treatment group compared to model control group ($P<0.01$-0.05, Table 2 and Figure 2).

**Table 2** Effect of garlicin on expression of bcl-2 and bax and AI in rats with experimental UC (mean±SD, $n$ = 10)

| Group | Bcl-2 | Bax | AI |
|---|---|---|---|
| Normal control | 9.8±1.4 | 30.1±3.2 | 20.8±0.7 |
| Model control | 41.0±2.5[d] | 9.9±0.8[d] | 2.3±1.6[d] |
| Garlicin (10.0 mg/kg) | 22.4±0.8[a] | 11.2±4.3 | 9.8±1.3[a] |
| Garlicin (30.0 mg/kg) | 10.2±2.5[b] | 13.1±2.5 | 15.3±0.6[b] |

[a]$P<0.05$, [b]$P<0.01$ *vs* model control; [d]$P<0.01$ *vs* normal control.

## DISCUSSION

The term "apoptosis" describes the change of morphology different from cell necrosis. Hallmarks of apoptosis include chromatin condensation, nuclear segmentation, cytoplasmic shrinkage, blebbing, and formation of apoptotic bodies[18]. Bcl-2 is a suppressor gene of apoptosis, which was found from follicular B cell lymphoma with *t*(14,18) chromosome malposition[19]. Orientating as 18q21, bax, and bcl-2 are homologous proteins, and bax is an induction gene of apoptosis. Bcl-2 and bax can exist in the form of homodimer and form heterodimer too. When the expression of bax increases, the homodimer of bax–bax can induce apoptosis. When the expression of bcl-2 increases, bax can combine with bcl-2 to form more stable heterodimers which can inhibit apoptosis. The ratio of bcl-2/bax can regulate apoptosis[20].

The important pathological change in UC are inflammatory damage of intestine, during which a large number of inflammatory cells infiltrate the intestinal wall and activate continuously[21]. Apoptosis of the lymphocytes has a close relation with UC, and apoptosis of lymphocytes in colonic mucosa is the main reason for the development of UC[6,22-24]. This experiment has proved that apoptosis of lymphocytes is delayed in UC, which is consistent with previous reports[2,3,20]. The positive expression of Bcl-2 in lymphocytes increased while that of bax was relatively rare, which is in agreement with previous reports[20]. Garlicin is a kind of chemical compound extracted from garlic corn. It has many biological activities against inflammation, fungi, anti-oxidant, etc.[9-11].

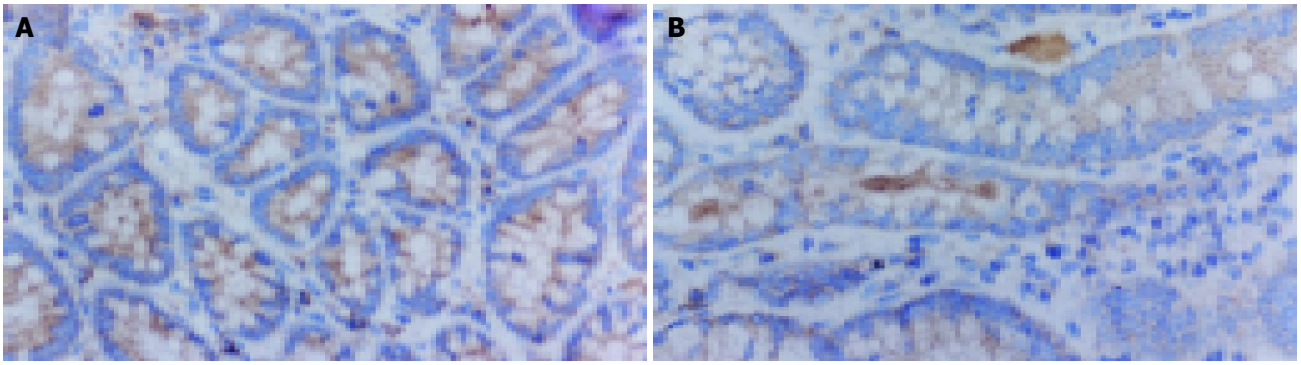


**Figure 1** Immunohistochemical staining of bcl-2 in tissue sections of model control group (**A**) and garlicin treatment group (**B**).

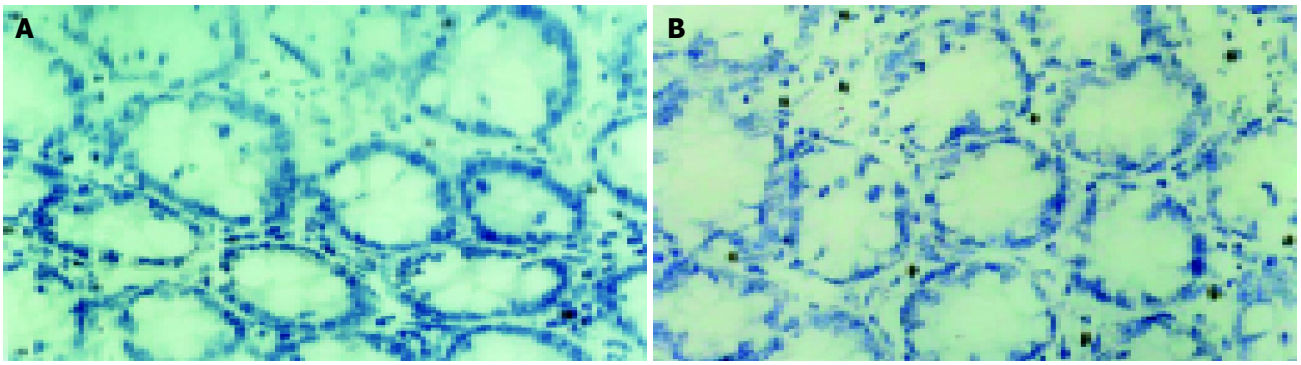


**Figure 2** TUNEL staining in tissue sections of model control group (**A**) and garlicin treatment group (**B**).

It can protect colonic mucosa against damage. In this study, the apoptosis of lymphocytes increased in garlicin treatment group and the positive expression of bcl-2 in lymphocytes decreased, suggesting that garlicin has protective effects on UC by promoting apoptosis of lymphocytes and reducing expression of bcl-2 protein.

We observed the effects of garlicin on the expression of bcl-2 and bax in lymphocytes in rat model of UC induced by TNBS. The results showed that the CMDI of garlicin treatment group was obviously lower than that of the model control group, indicating that the garlicin can prevent and cure UC. At the same time, the apoptosis of lymphocytes increased in garlicin treatment group, showing that the protective effects of garlicin in rats with UC are related with the induction of apoptosis of lymphocytes. The results of this experiment may open up a new way for the treatment of UC.

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