Original Article

Effects of Shu Bu Wenshen Guchang recipe on intestinal injury and the TLR4/NF-κB signaling pathways in mice with irinotecan-induced delayed-type diarrhea

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Background: Irinotecan (also known as CPT-11) is a topoisomerase I inhibitor that is primarily used for the treatment of advanced colorectal cancer. CPT-11 and its active metabolite SN-38 can directly damage intestinal mucosal cells. In addition, CPT-11 can activate the Toll-like receptor 4 (TLR4) inflammasome/nuclear factor kappa-B p65 (NF-κB p65) pathway, ultimately leading to intestinal inflammation-related injury. Shu Bu Wenshen Guchang recipe (SBWGR) has the spleen and kidneys. Herein, we investigated the effects of SBWGR on intestinal injury and the TLR4/NF-κB signaling pathways in mice with CPT-11-induced delayed-type diarrhea, aiming to provide evidence for the treatment of CPT-11-induced delayed-type diarrhea.

Methods: Thirty tumor-bearing mice were divided into normal control, model control, octreotide, low dose SBWGR, and high dose SBWGR groups, with 6 mice in each group. After successful modelling of delayed diarrhea, the normal and model control groups were given equal amounts of saline for 5 consecutive days, and the other three groups gave the corresponding intra-drug administration. Body weight, tumor size, Chiu score, intestinal ischemia and reperfusion injury, and disease activity index (DAI) were recorded in each group. The levels of intestinal interleukin-1β (IL-1β), IL-18, and tumor necrosis factor-α (TNF-α) were measured by an enzyme-linked immunosorbent assay (ELISA). Intestinal TLR4 and NF-κB p65 levels were measured by reverse transcription-polymerase chain reaction (RT-PCR) and protein blotting.

Results: The weight of octreotide and kidney was higher than the control group ($P<0.05$); The tumor volume comparison of the model control group, octreotide group, warm kidney intestine low dose group, and warm kidney intestine high dose group were not significantly different ($P>0.05$). Octreotide group, intestinal Chiu score, diarrhea score, DAI level, intestinal inflammatory cytokines, IL-1β, IL-18 and TNF-α intestinal level, intestinal TLR4, NF-κB p65 mRNA protein expression levels were significantly lower than those of the model control group ($P<0.05$), and the amount of the treatment group was increased ($P<0.05$).

Conclusions: SBWGR exerts a prominent protective effect on intestinal damage caused by CPT-11-induced delayed-type diarrhea, which may be achieved by inhibiting the activation of the intestinal TLR4/NF-κB signaling pathway.

Keywords: Shu Bu Wenshen Guchang recipe (SBWGR); delayed-type diarrhea; Toll-like receptor 4 (TLR4); nuclear factor kappa-B (NF-κB)

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Introduction

Irinotecan (also known as CPT-11), a semisynthetic camptothecin derivative, is a topoisomerase I inhibitor that is primarily used for the treatment of advanced colorectal cancer. However, CPT-11-induced intestinal mucosal damage and delayed-type diarrhea have notably impeded the clinical application of this chemotherapeutic drug (1). The overall incidence of clinically significant diarrhea caused by CPT-11 therapy is up to 87%, of which the incidence of grade 3–4 diarrhea was 30–40% (2). Persisting diarrhea may lead to excessive dehydration, electrolyte loss, acid-base disturbance, shock, and even death (2).

CPT-11 and its active metabolite SN-38 can directly damage intestinal mucosal cells. In addition, CPT-11 can activate the toll-like receptor 4 (TLR4) inflammasome/nuclear factor kappa-B p65 (NF-κB p65) pathway, which in turn increases the expressions of pro-inflammatory cytokines such as cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE2), thereby increasing the levels of inflammatory factors, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), and eventually leading to intestinal inflammation-related injuries (3,4). TLR4 inflammasomes are multiprotein complexes that are involved in infection, inflammation, and autoimmune processes. TLR4 plays a key role in innate immunity by producing pro-inflammatory cytokines; TLR4 activation triggers gastrointestinal inflammation and leads to the injury of intestinal epithelial cells, thereby reducing the integrity of the intestinal epithelium and leading to epithelial erosions (5,6).

According to traditional Chinese medicine (TCM), the functions of the spleen and stomach are inherently deficient in cancer patients; when the emotions are blocked and the liver qi is stagnant, the liver-wood suppresses the spleen-earth. CPT-11 aggravates damage to the spleen and stomach, resulting in failure of the spleen to ascend clearness and also stomach failure to descend turbidity. Due to the deficiency of the kidney yang and the decline of vital gate fire, the spleen-earth cannot be warmed, leading to qi deficiency in both the spleen and kidneys (7). Shu Bu Wenshen Guchang recipe (SBWGR) can invigorate the spleen, soothe the liver, warm the spleen and kidney, strengthen the intestines, and stop diarrhea, thereby exerting a dual effect of tonifying both the spleen and kidneys. Recent studies have shown that SBWGR exerts anti-inflammatory, antioxidant, and anti-tumor effects. SBWGR also protects the intestinal mucosal barrier in rat models of MTX-induced enteritis by inhibiting the activation of NF-κB and p38 mitogen-activated protein kinase (MAPK) (8,9).

Herein, we investigated the effects of SBWGR on intestinal injury and the TLR4/NF-κB signaling pathways in mice with CPT-11-induced delayed-type diarrhea, aiming to provide evidence for the treatment of CPT-11-induced delayed-type diarrhea. We present the following article in accordance with the ARRIVE reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-2145/rc).

Methods

Main equipment and reagents

Mouse colon cancer CT26 cell line cells (Shanghai Guyan Industrial Co., Ltd., Shanghai, China; Lot No. 41596); Octreotide (Merck Biotechnology, Germany; Batch number: df-4854), mirVana miRNA isolation kit (Ambion Biotechnology, USA; Lot No. 63254.69), PrimeScript 1st strand cDNA Synthesis Kit (Dalian Baori Biotechnology, Dalian, China; Batch number: bj-63306); Power SYBR Green PCR Master Mix (Applied Biosystems Biotechnology, USA; Batch number: op-203.25); ABI 7500 (GM), Protease inhibitors (Sigma Biotechnology, Germany; Batch ot.: 632659.9); BCA protein determination kit (Beyotime Biotechnology, Shanghai, China; Batch number: as63695); TLR4, NF-κB p65 primary (1:500, 1:1,000; Thermo Fisher, USA; ab41901,69354), rabbit monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1,000; Thermo Fisher, USA; Batch number: ab128015), horseradish peroxidase secondary antibody (1:2,000; Thermo Fisher, USA; Batch number: ab128015), ECL Plus Western Blotting System (Thermo Fisher, USA).

Experimental animals

Forty BALB/c mice were provided by Hunan Jack Jingdong Experimental Animal Co., Ltd. [License No. scxk (Xiang) 2019-0004, Certificate No. scxk (Xiang) 2019-0004]. Mice were kept in ordinary animal houses: temperature (20–24 ℃), light (12:12 light-dark cycle), humidity 60–70%. A protocol was prepared before the study without registration. The study was approved by the Ethics Committee of our hospital (approval number: 2022-DWSY-LZL), and the experiments were performed strictly according to National Guidelines for the Care and Use of Animals.

Scheme of experimental groups

Two healthy BALB/c mice were taken, and mouse colon cancer CT26 cells were resuscitated, and inoculated with 5,106/only mice back, and tumor bodies were formed after 7–10 days.

Mice with well-grown tumors and no ulceration on the surface were sacrificed. Next, the areas that needed to be operated on around the tumor were disinfected and the skin was cut open and the fascia was peeled off, so as to completely harvest the tumor tissue and place it in a sterile container. The tumor tissue was cut into small pieces (2–3 mm^3), ground evenly with sterile glass, and then placed into a sterile container. Normal saline was added at a ratio of 1:3 or 1:4 to prepare the tumor cell suspension. After the cell survival rate reached >95%, as determined by 0.4% trypan blue staining, the tumor cells were inoculated on the backs of 30 healthy female BALB/c mice (0.2 mL/mouse, about 5×10^6 cells/mouse).

When the tumors grew to about 100 mm^3 in all of the mice, they were divided into a normal control group, a model control group, an octreotide group, a low-dose SBWGR group, and a high-dose SBWGR group according to their body weight using a random number table. In addition to the normal control group, CPT-11 350 mg/kg was injected in the model control group, octreotide group, low-dose group, and high-dose kidney solid bowel square in C/200 group. (a pilot experiment showed that the LD_{50} of CPT-11 to mice was 1,400 mg/kg, with 1/4 LD_{50} as the administrated dose), and the intraperitoneal injection volume was 2.0 mL/100 g. As a result, 60% of the mice had mild diarrhea immediately after the injection, 30% progressed to moderate diarrhea After 24 h, and 10% of the mice developed severe diarrhea after 24 h. Thus, the mouse models of CPT-11-induced delayed-type diarrhea were successfully established.

Subsequently, the octreotide group (octreotide 100.0 mg/kg; the ED_{50} of octreotide on CPT-11-induced delayed-typed diarrhea mice was 200 mg/kg in the pilot experiment, and 1/2 ED_{50} was used as the administration dose), low-dose SBWGR group (SBWGR 100.0 mg/kg; the ED_{50} of SBWGR on CPT-11-induced delayed-type diarrhea mice was 400 mg/kg in the pilot experiment, and 1/4 ED_{50} was the administration dose), and high-dose SBWGR group (SBWGR 200.0 mg/kg; 1/2 ED_{50} was the administration dose) were intragastrically administered with the corresponding drug (volume: 1.0 mL/100 g). The normal control and model control groups were given an equal volume of normal saline for five consecutive days.

Tissue sampling

Mice in each group were fasted for 12 hours after the last administration. Under anesthesia with 0.4% sodium pentobarbital, a piece of colon content (about 2 g) was harvested, quickly placed into a sterile stool sampler, and immediately frozen at a –80 ℃ refrigerator for further analyses. Then, 5 cm of colon tissue was collected from each group and washed with PBS solution. Specimens used for pathological observation were fixed in formalin for 48 h.

Recording of the body weight, intestinal mucosal ischemia-reperfusion injury Chiu score, diarrhea score, and disease activity index (DAI) of mice in each group

The body weights of the mice in each group were recorded upon completion of each experiment. The long and short diameters of the tumors were measured using vernier calipers, the tumor volume was calculated, and growth curves were drawn by averaging each group. The following formula was applied: tumor volume (V) = ab2/2, where "a" is the largest diameter of the tumor and "b" is the smallest diameter of the tumor. The sum of the volumes was calculated in cases of multiple tumors.

The mice in each group were sacrificed, the intestinal tissue was observed pathologically, and the Chiu score was obtained. The scoring criteria were as follows: 0, mucosa with normal villi; 1, development of the subepithelial Gruenhagen's space, usually at the villus apex, frequently associated with capillary congestion; 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; 3, massive epithelial lifting down the sides of the villi; 4, denuded ill with lamina propria and dilated capillaries exposed, and increased cellularity of lamina propria may also be noted; and 5, digestion and disintegration of lamina propria as well as hemorrhage and ulceration.

The body weights of all mice in each group were recorded, and diarrhea and rectal bleeding were observed. Accordingly, the DAI score was analyzed by combining the scores for body weight loss (0: none; 1: 1–5%; 2: 5–10%; 3: 11–15%; 4: >15%), stool consistency (0, normal; 2, loose stools; 4, watery diarrhea), and rectal bleeding (0, normal; 2, slight bleeding; 4, gross bleeding). DAI was calculated as a total score (body weight decrease + stool consistency + rectal bleeding) divided by three.

Pathological observation of intestinal mucosa

After fixation of the mouse colon tissue with formalin, the parts to be observed were trimmed and then placed in tissue embedding cassettes. Subsequently, they were rinsed, dehydrated, and immersed in a solution of melted paraffin. Following condensation of the paraffin into blocks, the blocks were fixed on the microtome and cut into 3–5-μm sections, which were placed in hot water until the sections were fully unfolded. These sections were picked up using a glass slide and dried in an incubator. After HE staining, the morphology and pathology of the mouse colonic mucosa were observed.

Detection of inflammatory markers in intestinal tissue

The tissue specimens were cut, weighed, and then maintained at a temperature of 2–8 ℃. A certain amount of PBS buffer was added, and the samples were fully homogenized using a homogenizer. After centrifugation at 2,000 rpm for 20 min, the supernatant was carefully collected. Enzyme-linked immunosorbent assay (ELISA) was performed by referring to the manufacturer's instructions to detect the expression levels of pro-inflammatory factors including IL-1β, IL-18, and TNF-α.

Detection of intestinal TLR4 and NF-κB p65 gene expression levels in mice with severe acute enteritis

RNA was isolated from tissue or cells using the mirVana miRNA Isolation Kit according to the manufacturer's instructions. First-strand cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit. Quantitative polymerase chain reaction (qPCR) was performed using the Power SYBR Green PCR Master Mix. The primer sequences for TLR4 were as follows: 5'-TCGAGCTAGCTAGCTAGCTGATCGATCGATCGATCGCC-3' (forward) and 5'-TGCGGCTAGCTAGCTAGCTGATCGATCGATCGATCGATGC-3' (reverse). The primer sequences for NF-κB p65 were as follows: 5'-TGGGCTAGCTAGTCGATCGATCGATCGTAGCTAGCTAGCA-3' (forward) and 5'-TGGGCTAGTCGATCGTAGCTAGCTAGCTAGCTAGTCGATC-3' (reverse). The primer sequences for GAPDH were as follows: 5'-TCGAGGCTAGCTGTCGATCGATCGATCGTAGTC-3' (forward) and 5'-TGGTCGTAGCTAGTGTGTCGTAGCTAGCTGATGC-3' (reverse). A 20-μL final volume contained 10 μL of SYBR, 0.40 μmol/L of each primer, and 0.2±0.02 μg of cDNA template. The thermal cycling conditions were as follows: pre-denaturation at 95 ℃ for 3 min, followed by 40 cycles of denaturation at 95 ℃ for 5 s, annealing at 60 ℃ for 20 s, and extension at 72 ℃ for 20 s. PCR was performed using an ABI PRISM® 7500 FAST Sequence Detection System, with GAPDH as an internal control. The relative expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Detection of intestinal TLR4 and NF-κB p65 protein expression levels in mice with severe acute enteritis

Protein extracts were prepared in lysis buffer containing protease inhibitors, and protein concentrations were measured using a BCA Protein Assay Kit. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk for 1 h and then incubated with primary antibodies overnight at 4 ℃. The primary antibodies, including TLR4 and NF-κB p65 mouse monoclonal antibodies and GAPDH rabbit monoclonal antibody, were applied first, and the membranes then were reacted with horseradish peroxidase-conjugated secondary antibodies (1:2,000) for 1 h.

Detection was performed on the ECL Plus Western Blotting System. The bands of TLR4 and NF-κB p65 on the membrane were measured by ImagePro Plus 6.0 software, and the protein expressions of TLR4 and NF-κB p65 were calculated according to the following formula: [TLR4/NF-κB p65 [integrated option density (IOD) value] of the experimental group/GAPDH (IOD value) of the experimental group]/[TLR4/NF-κB p65 (IOD value) in the normal control group/GAPDH (IOD value) in the normal control group].

Statistical analysis

Data entry and statistical analyses were performed using Epidata and SPSS19.0 software. Measurement data were expressed as mean ± standard deviation (SD). Data were tested for normality first; normally distributed data were analyzed using the LSD-q pairwise test, while non-normally distributed data were transformed before the LSD-q pairwise test. The significance level was set at 0.05.

Table 1 Effects of SBWGR on body weight and tumor size in mice with CPT-11-induced delayed-type diarrhea (mean ± SD)

Group	n	Dose (mg/kg)	Body weight (g)	Tumor size (mm^3)
Normal control group	6	–	25.36±1.32	126.36±13.96
Model control group	6	–	19.52±1.58*	102.72±13.84*
Octreotide group	6	100	29.54±1.39$^{\#}$	104.47±15.74*
Low-dose SBWGR group	6	50	26.48±1.49$^{\#\&}$	103.34±16.57*
High-dose SBWGR group	6	200	30.25±1.63$^{\#\$}$	99.27±17.52*

*P<0.05, compared with the normal control group; $^{\#}$P<0.05, compared with the model control group; $^{\&}$P<0.05, compared with the octreotide group; $^{\$}$P<0.05, compared with low-dose SBWGR group. SBWGR, Shu Bu Wenshen Guchang recipe; SD, standard deviation.

Table 2 Effects of SBWGR on the intestinal Chiu score, diarrhea score, and DAI in mice with CPT-11-induced delayed-type diarrhea (mean ± SD)

Group	n	Dose (mg/kg)	Intestinal Chiu score (points)	Diarrhea score (points)	DAI (points)
Normal control group	6	–	0.57±0.49	0.00±0.00	0.00±0.00
Model control group	6	–	4.54±0.38*	2.15±0.67*	3.54±0.42*
Octreotide group	6	100	1.69±0.36$^{\#}$	0.85±0.29$^{\#}$	1.52±0.42$^{\#}$
Low-dose SBWGR group	6	50	3.43±0.41$^{\#\&}$	1.68±0.27$^{\#\&}$	2.69±0.46$^{\#\&}$
High-dose SBWGR group	6	200	1.66±0.99$^{\#\$}$	0.89±0.27$^{\#\$}$	1.53±0.43$^{\#\$}$

*P<0.05, compared with the normal control group; $^{\#}$P<0.05, compared with the model control group; $^{\&}$P<0.05, compared with the octreotide group; $^{\$}$P<0.05, compared with low-dose SBWGR group. SBWGR, Shu Bu Wenshen Guchang recipe; DAI, disease activity index; SD, standard deviation.

Results

Effects of SBWGR on body weight and tumor size in mice with CPT-11-induced delayed-type diarrhea

As shown in *Table 1*, the body weights of the model control group mice were significantly lower than those of the normal control group mice (P<0.05), and were markedly higher in the octreotide and SBWGR groups than those in the model control group (all P<0.05). In addition, the body weight was notably higher in the high-dose SBWGR group than in the low-dose SBWGR group (P<0.05). Furthermore, it was considerably lower in the low-dose SBWGR group than in the octreotide group (P<0.05) but exhibited no significant difference between the high-dose SBWGR and octreotide groups (P>0.05).

The tumor size in the model control group was markedly smaller than that in the normal control group (P<0.05). However, it was not significantly different among the model control, octreotide, low-dose SBWGR, and high-dose SBWGR groups (all P>0.05).

Effects of SBWGR on the intestinal Chiu score, diarrhea score, and DAI in mice with CPT-11-induced delayed-type diarrhea

As shown in *Table 2*, the Chiu score, diarrhea score, and DAI were significantly higher in the model control group than in the normal control group (all P<0.05). These indicators were markedly lower in the octreotide and SBWGR groups than those in the model control group (all P<0.05) and notably decreased in the high-dose SBWGR group (all P<0.05). Moreover, these indicators were substantially higher in the low-dose SBWGR group than in the octreotide group (all P<0.05) but showed no significant difference between the high-dose SBWGR and octreotide groups (all P>0.05).

Effects of SBWGR on intestinal pro-inflammatory factors including IL-1β, IL-18, and TNF-α in mice with CPT-11-induced delayed-type diarrhea

As shown in *Table 3*, the levels of intestinal inflammatory

Table 3 Effects of SBWGR on intestinal pro-inflammatory factors including IL-1β, IL-18, and TNF-α in mice with CPT-11-induced delayed-type diarrhea (mean ± SD)

Group	n	Dose (mg/kg)	IL-1β (pg/mL)	IL-18 (pg/mL)	TNF-α (pg/mL)
Normal control group	6	–	65.95±17.52	105.63±26.63	43.65±15.69
Model control group	6	–	365.25±26.52*	452.69±32.65*	259.54±84.26*
Octreotide group	6	100	154.47±37.52#	165.98±39.54#	165.25±35.14#
Low-dose SBWGR group	6	50	206.65±41.52#&	235.58±52.14#&	198.54±38.48#&
High-dose SBWGR group	6	200	162.36±35.64#$	172.02±42.63#$	167.29±37.25#$

*P<0.05, compared with the normal control group; #P<0.05, compared with the model control group; &P<0.05, compared with the octreotide group; $P<0.05, compared with low-dose SBWGR group. SBWGR, Shu Bu Wenshen Guchang recipe; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-alpha; SD, standard deviation.

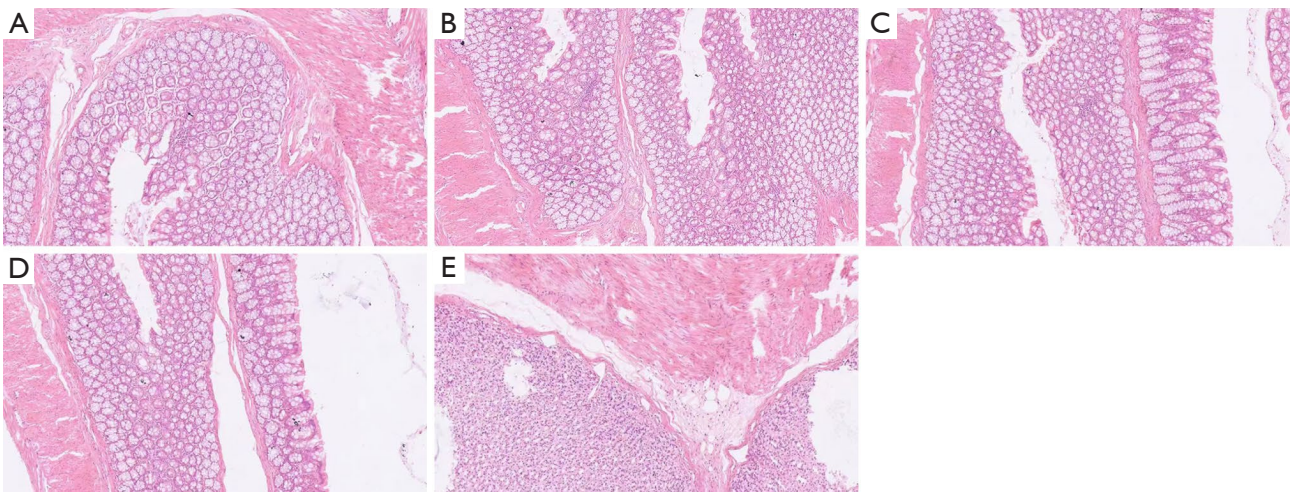

Figure 1 Effects of SBWGR on the intestinal structures in mice with CPT-11-induced delayed-type diarrhea (HE, ×400). HE (Tissue stained sections). (A) The normal control group; (B) the model control group; (C) the octreotide group; (D) the low-dose SBWGR group; (E) the high-dose SBWGR group. SBWGR, Shu Bu Wenshen Guchang recipe; HE, hematoxylin and eosin.

cytokines IL-1β, IL-18, and TNF-α were significantly higher in the model control group than in the normal control group (all P<0.05). They were also considerably lower in the octreotide and SBWGR groups than in the model control group (all P<0.05) and significantly decreased in the high-dose SBWGR group (all P<0.05). Furthermore, these indicators were also notably higher in the low-dose SBWGR group than in the octreotide group (all P<0.05) but showed no significant difference between the high-dose SBWGR and octreotide groups (all P>0.05).

Effects of SBWGR on the intestinal structures in mice with CPT-11-induced delayed-type diarrhea

As shown in *Figure 1*, mice in the normal control group had normal intestinal structures. In the model control group, the intestinal mucosa was congested, the intestinal mucosal epithelial cells fell off, and obvious inflammatory cell infiltration (mainly neutrophils and eosinophils) was visible. In both the octreotide and high-dose SBWGR groups, the intestinal structure tended to be normal, the

Table 4 Effects of SBWGR on intestinal TLR4 and NF-κB p65 mRNA expressions in mice with CPT-11-induced delayed-type diarrhea (mean ± SD)

Group	n	Dose (mg/kg)	TLR4 mRNA	NF-κB p65 mRNA
Normal control group	6	–	0.64±0.14	0.90±0.10
Model control group	6	–	4.36±0.16*	3.73±0.19*
Octreotide group	6	100	1.48±0.21#	1.26±0.14#
Low-dose SBWGR group	6	50	3.63±0.18#&	2.96±0.14#&
High-dose SBWGR group	6	200	1.36±0.69#$	1.26±0.152#$

*P<0.05, compared with the normal control group; #P<0.05, compared with the model control group; &P<0.05, compared with the octreotide group; $P<0.05, compared with low-dose SBWGR group. SBWGR, Shu Bu Wenshen Guchang recipe; TLR4, Toll-like receptor 4; NF-κB, nuclear factor kappa-B; SD, standard deviation.

Table 5 Effects of SBWGR on the intestinal TLR4 and NF-κB p65 protein expressions in mice with CPT-11-induced delayed-type diarrhea (mean ± SD)

Group	n	Dose (mg/kg)	TLR4 (/GAPDH)	NF-κB p65 (/GAPDH)
Normal control group	6	–	0.36±0.12	0.29±0.09
Model control group	6	–	1.26±0.24*	1.35±0.30*
Octreotide group	6	100	0.51±0.10#	0.49±0.17#
Low-dose SBWGR group	6	50	0.87±0.21#&	0.75±0.24#&
High-dose SBWGR group	6	200	0.53±0.19#$	0.50±0.18#$

*P<0.05, compared with the normal control group; #P<0.05, compared with the model control group; &P<0.05, compared with the octreotide group; $P<0.05, compared with low-dose SBWGR group. SBWGR, Shu Bu Wenshen Guchang recipe; TLR4, Toll-like receptor 4; NF-κB, nuclear factor kappa-B; SD, standard deviation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

intestinal mucosal congestion and shedding of intestinal mucosal epithelial cells were significantly alleviated, and the infiltration of inflammatory cells was reduced. However, although the intestinal structures in the low-dose SBWGR group were also improved, there was still obvious congestion and inflammatory cell infiltration.

Effects of SBWGR on the intestinal TLR4 and NF-κB p65 mRNA expressions in mice with CPT-11-induced delayed-type diarrhea

As shown in *Table 4*, the intestinal mRNA expression levels of TLR4 and NF-κB p65 were significantly higher in the model control group than in the normal control group (both P<0.05). They were also significantly lower in the octreotide and SBWGR groups than in the model control group (all P<0.05), and were significantly decreased in the high-dose SBWGR group (all P<0.05). In addition, these indicators were significantly higher in the low-dose SBWGR group than in the octreotide group (both P<0.05) but showed no significant difference between the high-dose SBWGR and octreotide groups (both P>0.05).

Effects of SBWGR on the intestinal TLR4 and NF-κB p65 protein expressions in mice with CPT-11-induced delayed-type diarrhea

As shown in *Table 5* and *Figure 2*, the intestinal protein expression levels of TLR4 and NF-κB p65 were significantly higher in the model control group than in the normal control group (both P<0.05). They were also markedly lower in the octreotide and SBWGR groups than in the model control group (all P<0.05), and were notably decreased in the high-dose SBWGR group (all P<0.05). Additionally, these indicators were also significantly higher in the low-dose SBWGR group than in the octreotide group (both P<0.05) but showed no significant difference between the high-dose SBWGR and octreotide groups (both P>0.05).

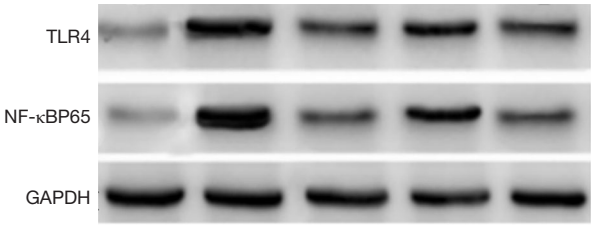

Figure 2 Effects of SBWGR on the intestinal TLR4 and NF-κB p65 protein expressions in mice with CPT-11-induced delayed-type diarrhea. TLR4, Toll-like receptor 4; NF-κB, nuclear factor kappa-B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SBWGR, Shu Bu Wenshen Guchang recipe.

Discussion

TCM has been increasingly used to control cancer-related side effects and reduce treatment-related toxicities. Several TCM extracts and compounds have shown a considerable ability to inhibit CPT-11-induced intestinal toxicities. Phase I/II trials have demonstrated that SBWGR can combat gastrointestinal toxicities by enhancing multiple anti-cancer mechanisms of other therapies. Therefore, SBWGR may be a promising adjuvant therapy when used in combination with CPT-11.

SBWGR is an empirical recipe developed by Dr. Wang Renqiang that comprises Caulis Perillae, Pericarpium Citri Reticulatae, fried Fructus Aurantii, Radix Pseudostellariae, fried Rhizoma Atractylodis Macrocephatae, Poria cocos, Radix Morindae Officinalis, Herba Epimedii, Lobed Kudzuvine root, Rosa laevigata Michx, Radix Aucklandiae, and Rhizoma Coptidis, and has been widely used in the treatment of gastrointestinal diseases, especially diarrhea (10). According to TCM theories, CPT-11-induced delayed-type diarrhea is caused by liver stagnation, spleen deficiency, and yang deficiencies in the spleen and kidneys. SBWGR can tonify both the spleen and kidneys. Lobed Kudzuvine root and Rhizoma Coptidis are two key components in the recipe, and both puerarin and berberine are recognized as bioactive compounds with significant anti-diarrheal activity (11).

According to TCM theory and herbal properties, SBWGR can effectively suppress diarrhea, thereby relieving gastrointestinal pain. In addition, several chemicals such as puerarin, berberine, and baicalin have well-documented anti-cancer effects. Therefore, we propose that SBWGR may be a promising and useful adjunct in the CPT-11 regimen, as it can alleviate the associated intestinal toxicity while maintaining and promoting its anti-cancer effect. Our present study showed that SBWGR could significantly prevent weight loss in mice with CPT-11-induced delayed-type diarrhea, and high-dose SBWGR had similar efficacy to that of the classic drug octreotide. The tumor size was significantly reduced in both the low- and high-dose SBWGR groups, further demonstrating the anti-cancer effect of this recipe.

SBWGR has been shown to reduce the apoptosis of intestinal mucosal cells, protect intestinal mucosal cells, and promote the proliferation and differentiation of intestinal stem cells, thereby repairing the damaged intestinal mucosa. Also, it can effectively inhibit CPT-11-induced multiplication and differentiation of multiple inflammatory markers and alleviate CPT-11 chemotherapy-induced diarrhea (12). In addition, some monomers and active ingredients in SBWGR, such as Muxiang and hesperidin, have also been shown to reduce the expressions of some inflammatory factors (e.g., TNF-α) and inhibit the apoptosis of intestinal epithelial cells, thus alleviating the toxic damage of CPT-11 to the intestinal tract (13).

In our present study, the levels of intestinal inflammatory cytokines (IL-1β, IL-18, and TNF-α) in the SBWGR group were significantly lower than those in the control group (all $P<0.05$) and markedly decreased in the high-dose SBWGR group (all $P<0.05$). In both the octreotide and high-dose SBWGR groups, pathological examination showed that the intestinal structure tended to be normal, the intestinal mucosal congestion and the shedding of intestinal mucosal epithelial cells were significantly alleviated, and the infiltration of inflammatory cells was reduced. However, in the low-dose SBWGR group, although the intestinal structures were also improved, there were still obvious congestion and inflammatory cell infiltration. These findings again confirmed that SBWGR exerts a prominent protective effect against intestinal injury in patients with CPT-11-induced delayed-type diarrhea.

DNA damage is one of the main mechanisms through which CPT-11 kills cells. A study has shown that DNA damage can induce oxidative inflammation. NF-κB is a nuclear transcription factor that is involved in various inflammatory responses, cellular oxidative stress, cellular transformation, tumor invasion, and drug resistance. (14). Normally, NF-κB is a dimer mainly composed of the NF-κB p65 and NF-κB p50 subunits, which remain inactive in the cytoplasm (15). When cells are stimulated by internal and external factors such as TLR4, TNF-α, and/or CPT-11, NF-κB p65 is released from the NF-κB transcription

complex and translocated to the nucleus, thereby activating transcription in a series of inflammatory genes (IL-2, IL-6, ICAM-1, E-selectin). *In vitro* cell studies have found that CPT-11 can induce DNA damage in intestinal IEC-6 cells, leading to increased intracellular ROS and TLR4 levels and NF-κB activation, which disrupts intestinal redox balance, causes intestinal mucosal damage, and induces cellular apoptosis (16,17).

Free radical scavenging and anti-inflammatory activities are two key pharmacological effects of SBWGR. In rat models of diabetic gastroparesis, SBWGR was shown to improve symptoms associated with delayed gastric emptying, which may help to block the production of oxidative stress and inhibit TLR4/NF-κB signaling. In DSS-induced ulcerative colitis models, SBWGR increased the intestinal superoxide dismutase (SOD), glutathione (GSH), and glutathione S-transferase (GST), and down-regulated the levels of inflammatory factors such as MDA, IL-4, and IL-8 (18). SBWGR has a protective effect on MTX-induced enteritis in rats, which may be achieved by inhibiting the activation of NF-κB in intestinal mucosal cells and regulating the expression of inflammatory factors. SBWGR was shown to inhibit NF-κB activation in a 3-nitrobenzenesulfonic acid-induced colitis model. In addition, SBWGR inhibited IL-1β-induced NF-κB activation in human articular chondrocytes (19).

In our current study, the mRNA expressions and protein levels of both TLR4 and NF-κB p65 were significantly higher in the model control group than in the normal control group, suggesting that CPT-11 induced the high expression of TLR4 in mouse intestinal cells, which led to the nuclear translocation of NF-κB p65. After treatment with SBWGR (20), the mRNA expressions and protein levels of both TLR4 and NF-κB p65 in the SBWGR group were markedly lower than those in the model control group (all $P<0.05$) and further decreased in the high-dose SBWGR group (all $P<0.05$), suggesting SBWGR significantly decreased CPT-11-induced high expression of TLR4 and the nuclear translocation of NF-κB p65 in mouse intestinal cells. Since SBWGR suppresses a variety of intestinal inflammatory responses by regulating NF-κB, it is assumed that the inhibitory effect of SBWGR on intestinal injury in mice with CPT-11-induced delayed-type diarrhea is closely related to suppressing the activation of TLR4/NF-κB signaling pathways.

In conclusion, SBWGR exerts a prominent protective effect on intestinal damage caused by CPT-11-induced delayed-type diarrhea, which may be achieved by inhibiting the activation of the intestinal TLR4/NF-κB signaling pathway.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Ethics Committee of our hospital (approval number: 2022-DWSY-LZL), and the experiments were performed strictly according to National Guidelines for the Care and Use of Animals.

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