The protective effects of Xuebijing injection on intestinal injuries of mice exposed to irradiation

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Abstract
Background: Gastrointestinal (GI) injury is one of the most common side effects of radiotherapy. However, there is no ideal therapy method except for symptomatic treatment in the clinic. Xuebijing (XBJ) is a traditional Chinese medicine, used to treat sepsis by injection. In this study, the protective effects of XBJ on radiation-induced intestinal injury (RIII) and its mechanism were explored.

Methods: The effect of XBJ on survival of irradiated C57BL/6 mice was monitored. Histological changes including the number of crypts and the length of villi were evaluated by H&E. The expression of Lgr5+ intestinal stem cells (ISCs), Ki67+ cells, villin and lysozymes were examined by immunohistochemistry. The expression of cytokines in the intestinal crypt was detected by RT-PCR. DNA damage and apoptosis rates in the small intestine were also evaluated by immunofluorescence.

Results: In the present study, XBJ improved the survival rate of the mice after 8.0 and 9.0 Gy total body irradiation (TBI). XBJ attenuated structural damage of the small intestine, maintained regenerative ability and promoted proliferation and differentiation of crypt cells, decreased apoptosis rate and reduced DNA damage in the intestine. Elevation of IL-6 and TNF-α was limited, but IL-1, TNF-β and IL-10 levels were increased in XBJ-treated group after irradiation. The expression of Bax and p53 were decreased after XBJ treatment.

Conclusions: Taken together, XBJ provides a protective effect on RIII by inhibiting inflammation and blocking p53-related apoptosis pathway.

KEYWORDS
Xuebijing injection, Intestinal injury, Total body irradiation, Inflammation, Apoptosis

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1 | INTRODUCTION

Radiation-induced intestinal injury (RIII) is a severe side effect in tumor radiotherapy, especially in the patients with abdominal or pelvic malignancies. High doses of ionizing radiation (IR) cause acute injury to the epithelium of the small intestine and may even cause death within 10 days, which reflects the toxic reaction of the gastrointestinal (GI) tract. Intestinal epithelial cells maintain intestinal function through rapid self-renewal, but epithelial cells lack the ability to proliferate and homeostasis of the intestine is mainly supported by intestinal stem cells (ISCs) located at the base of the crypt. IR inhibits the proliferation of small intestinal epithelial cells, leading to necrosis of crypt cells, in turn reducing the number of crypts, increasing apoptotic cells, and destroying the structural integrity of villi. In contrast to IR-induced hematopoietic injuries, which can be prevented by bone marrow transplantation and treated with cytokines, the new standard for the treatment of radiation enteritis does not mention any effective therapeutic drugs. Therefore, it is very important to develop and find new drugs and targets for the treatment of radiation enteritis.

RIII prevention and treatment research is mainly focused on natural antioxidants (such as vitamins and amino acids), known body protectors, newly synthesized compounds, and biotechnological methods. A large number of studies have shown that Chinese medicines or extracts may alleviate radiation damage, and also have protective effects on radiation enteritis, including menthol and ginseng, which are attracting increasing attention due to their low toxicity and are used for radiation protection and treatment. Therefore, Chinese medicine compounds may be effective in RIII protection and therapy.

The traditional Chinese medicine XBJ was found by screening the prescriptions from 36 groups of traditional Chinese medicine formula under the guidance of the theory of Chinese medicine. Pharmacological studies have shown that safflower, salvia miltiorrhiza, red peony root, angelica and Chuanqiong – components of XBJ – can act on the apoptosis pathway of cells and have anti-inflammatory effects. XBJ has been approved by the State Food and Drug Administration (SFDA) of China for clinical treatment of sepsis. XBJ has also been used to treat radiation-related diseases. Our prior study has shown the role of XBJ in preventing IR-induced hematopoietic damage in irradiated mice. In this study, we further explored the radioprotective effects of XBJ on RIII and discuss the mechanism.

In summary, we evaluated the ability of XBJ to prevent RIII by measuring the survival rate and changes of cytokines in the small intestine. We found that XBJ may have a protective effect on RIII by inhibiting inflammation and blocking the p53-dependent apoptosis pathway.

2 | METHODS

2.1 | Animals

Male C57BL/6 mice aged 6-8 weeks were purchased from Beijing HFK Bioscience Co., Ltd (Beijing, China). The mice were in good health and were housed in a certified animal facility of the Institute of Radiation Medicine of the Chinese Academy of Medical Sciences (IRM-CAMS). They were provided with sterile water and autoclaved rat breeding feed to exclude the effects of bacteria and other effects. All experiments were carried out according to the NIH Laboratory Animal Care and Use Guidelines and obtained IRM-CAMS animal ethics approval (Permit Number 2019-0002).

2.2 | Irradiation treatment and XBJ administration

Mice were randomly divided into three groups: control, IR, and IR+XBJ. The IR+XBJ group were intraperitoneally injected with 0.4 ml/kg-day XBJ for 5 days in survival experiments and 3 days in the remaining experiments, and the control and IR groups were treated with normal saline solution. The XBJ preparation was purchased from Tianjin Chase Sun Pharmaceutical Co., Ltd as a liquid. The XBJ was diluted with saline and all mice were injected intraperitoneally with 200 μl of the solution. Mice were irradiated by gamma source (Gammacell-40) at a dose rate of 1.0 Gy/min. Mice were observed daily and bedding and water bottles were regularly changed post-irradiation to exclude the possibility of developing opportunistic infections.

2.3 | Intestinal crypt cell extraction

The method for extraction of intestinal crypt cells (ICCs) was as previously published. In short, the small intestines were rinsed with cold DPBS, cut into small pieces, and then placed in 2mM EDTA on an ice-bath shaker for 30 min. The fragments were then placed in cold dissociation buffer and vortexed. The solution was filtered and then centrifuged to obtain the crypt cells.

2.4 | Histological analysis

The small intestines were embedded in paraffin wax and then sectioned and stained with H&E and analyzed under a microscope. Then, the villi length and the crypt number were measured from HE-stained photographs.

2.5 | Immunohistochemistry analysis

The 4 μm-paraffin sections were dewaxed and dehydrated and followed by antigen retrieval. The sections were blocked with goat serum for 1 hour at room temperature (RT), and then incubated separately overnight at 4°C with primary antibodies, including anti-Lgr5 (Abcam), anti-Ki67 (Novus Littleton), anti-lysozyme (Abcam) and anti-villi (Abcam).

The sections were then incubated with secondary antibodies for 1 hour at RT, followed by the DAB kit (Sigma Aldrich) stain reaction. The pictures were captured and use IPP software was
used to quantify the positive cells. Five sections of small intestinal tissue were stained for each group, and Lgr5 and ki67 were counted in each of the five crypts, with three counts performed in each section.

2.6 RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from the small ICCs using TRIzol Reagent (Invitrogen). Then reverse transcription and real-time PCR were performed for analysis. Primer sequences are listed in Table 1.

2.7 TUNEL assay

The intestinal sections were stained using a TUNEL kit (Roche) according to the manufacturer’s instructions. The sections were photographed and statistically analyzed.

2.8 Western blot analysis

The ICCs were lysed to extract protein, and the protein concentration was quantified using a BCA assay kit (Beyotime). The membranes were incubated with primary antibodies including anti-Caspase3 (Proteintech), anti-Bax (Ruiyingbio) and β-tubulin antibodies (Proteintech). Proteins were detected with chemiluminescent reagents.

2.9 Immunofluorescence analysis

The paraffin wax sections were washed with PBS after antigen retrieval as described above. The sections were blocked for 30 minutes at RT, and then incubated with anti-caspase8 (CST), anti-caspase9 (CST), anti-γH2AX (BD biosciences) and anti-p53 (Ruiyingbio) at 4°C overnight. The sections were then incubated with fluorescent secondary antibody at 37°C for 1 hour avoiding light. Finally, DAPI was added, and the sections were sealed. Laser confocal photographs were taken followed by statistical analysis. Five sections of small intestinal tissue were taken for staining in each group, and three fields of view were selected for counting in each section.

2.10 Bone marrow cell micronucleus analysis

The thoracic vertebral bone marrow cell fluid was collected and air dried. The dried smear was then put into a methanol solution and fixed for 5 min. The fixed smear was placed into Giemsa application solution and stained for 20–30 min, after which it was immediately rinsed with 0.1 mol/L phosphate buffer. The stained sheet was dried with filter paper as soon as possible and then put into xylene for 5 minutes to form a transparent layer. The sample was dipped in optical resin glue and covered with a cover glass. Observations were performed with microscope and 1000 stained cells were counted per mouse.

2.11 Statistical analysis

Statistically analysis of the data was conducted using Graphpad Prism 8.0. The data were expressed as mean±standard deviation (SD), and p<0.05 indicated a significant difference. The difference between two groups was analyzed using a t test, and differences between the three groups was evaluated by one-way ANOVA.

3 RESULTS

3.1 XBJ improves the survival rate of mice following IR treatment

To evaluate the protective effect of XBJ injection on mice after IR, we determined the 30-day survival rate of mice after 7.5 Gy TBI. We previously showed that mice treated with IR+XBJ at 0.4 ml/kg had a higher survival rate than those treated with 0.13 ml/kg or and 1.2 ml/kg, which demonstrates that 0.4 ml/kg XBJ gave the best protection against IR-induced death. The radioprotective effects of XBJ on the survival rate of mice were thus evaluated by sham-irradiation or irradiation with 8.0 and 9.0 Gy γ-ray after receiving vehicle or 0.4 ml/kg dose XBJ (Figure 1). As shown in Figure 1A, there was 55.55% survival of the IR+XBJ group after 6 days of 8.0 Gy TBI, compared with only 11.11% survival in the IR group (p = 0.0233). The median number of survival days in the IR and IR+XBJ groups were 5.3 and 6.4 (p = 0.0325), respectively. As shown in Figure 1B, there was 46.67% survival in the IR+XBJ group after 6 days of 9.0 Gy TBI, compared with 6.67% in the IR group (p = 0.004). The median number of survival days in the IR and IR+XBJ groups were 4.9 and 6.4 (p = 0.0003), respectively. The above results demonstrated that XBJ effectively reduces

| Table 1 | qRT-PCR primers for the 5 genes evaluated |
|----------|-------------------------------------------|
| Gene symbol | Forward | Reverse |
| IL-6     | CCGAGAGGAGACTTCCACAGAG | TTTCCACAGATTTCCCAGAGA |
| IL-10    | TGGTGAACCCCGTCTCTAC | TGCCCTAGCCTCCCAAAGTA |
| TNF-β    | TCCCTGAACCATCCCTGAT | ATGTCCTGCTTCTCCCTG |
| TNF-α    | CCGTGAGGAGGAGGAACAT | GAAGAGTTGAGGTTGCTG |
| IL-1     | TGACTGGCCAAGATGAAG | CCCGAGTTTCCCAAGAAGA |

19 The radioprotective effects of XBJ on the survival rate of mice were thus evaluated by sham-irradiation or irradiation with 8.0 and 9.0 Gy γ-ray after receiving vehicle or 0.4 ml/kg dose XBJ (Figure 1). As shown in Figure 1A, there was 55.55% survival of the IR+XBJ group after 6 days of 8.0 Gy TBI, compared with only 11.11% survival in the IR group (p = 0.0233). The median number of survival days in the IR and IR+XBJ groups were 5.3 and 6.4 (p = 0.0325), respectively. As shown in Figure 1B, there was 46.67% survival in the IR+XBJ group after 6 days of 9.0 Gy TBI, compared with 6.67% in the IR group (p = 0.004). The median number of survival days in the IR and IR+XBJ groups were 4.9 and 6.4 (p = 0.0003), respectively. The above results demonstrated that XBJ effectively reduces

2.11 Statistical analysis

Statistically analysis of the data was conducted using Graphpad Prism 8.0. The data were expressed as mean±standard deviation (SD), and p<0.05 indicated a significant difference. The difference between two groups was analyzed using a t test, and differences between the three groups was evaluated by one-way ANOVA.
3.2 | XBJ relieves the damage to intestinal morphology in mice following IR treatment

To clarify the effect of XBJ on RIII, the small intestinal crypt-villi structure was observed using H&E staining as shown in Figure 2. After 9.0Gy TBI, the number of crypts was significantly reduced and the lengths of villi were shortened, and the structure significantly improved after XBJ treatment (Figure 2B-D). Compared to the control mice (25.67±1.437), the number of crypts in IR mice (15.34±0.909) decreased (p<0.005). XBJ treatment enhanced the number of surviving crypts (18.80±0.735, p<0.01). The villous height was significantly reduced in the IR group (410.9±8.32μm) compared with control group (568.1±11.71μm, p<0.005). The villous height was increased in XBJ-treated group (441.4±13.78μm) compared with the IR group (p<0.05). The expression of villi+C entersocytes was also affected by IR, with mice treated with XBJ exhibiting a significantly increase compared with the IR mice. Taken together, our data revealed that XBJ could protect mice from post-radiation damage to intestinal villus-crypt structure.

3.3 | XBJ maintains the expression of Lgr5 and Ki67 in the intestinal cells following IR treatment

To analyze the effect of XBJ on the proliferation and differentiation ability of crypt ISCs, Lgr5 and Ki67 were detected by immunohistochemistry. Lgr5+C ISCs are essential for the regeneration of the small intestine after IR and the expression of Ki67 is related to cell proliferation. As shown in Figure 3, the number of Lgr5+C
ISCs was significantly greater in the IR+XBJ group (6.30 ± 0.539) than in the IR group (2.30 ± 0.300, p < 0.01). Similarly, the number of Ki67+ proliferative cells in the XBJ-treated group (57.46 ± 1.287) was markedly greater than in IR mice (28.67 ± 1.457, p < 0.01). The difference in the expression of Lgr5 and Ki67 indicated that XBJ maintained the differentiation and proliferation capability of ISCs in mice after IR.

3.4 | XBJ reduces intestinal cell apoptosis following IR treatment

To analyze the effect of XBJ on intestinal cell apoptosis after IR, we assessed apoptosis using the TUNEL assay. The apoptosis cells were measured in small intestinal sections 3 days after 9.0 Gy TBI. As shown in Figure 4A,B, XBJ had a protective role in preventing RIII through inhibition of apoptosis. There were significantly fewer apoptotic cells in the XBJ-treated mice (51.00 ± 2.025) than in the IR mice (97.40 ± 2.135, p < 0.005). To further verify our finding, we also determined the apoptotic rate by analyzing the expression of Caspase-8 (13.75 ± 2.136) and Caspase-9 (23.20 ± 4.352) from the small intestinal sections of mice after IR. The IF images show the expression of apoptosis-related protein (Figure 4C,E). We found that the number of apoptotic cells in the small intestine of mice increased after IR (Caspase8, 6.40 ± 0.927; Caspase9, 9.20 ± 1.114; p < 0.05 Figure 4D,F). We also observed the expression of Caspase-3, and found that the expression increased after IR (Figure 6E). In contrast, apoptosis decreased after XBJ treatment. These results showed that XBJ could reduce the rate of apoptosis and protect the RIIIs.

3.5 | XBJ inhibits the inflammation of the small intestine following IR treatment

To evaluate the effect of XBJ on inflammation of the small intestine, we analyzed the expression of mRNA for inflammatory cytokine including IL-1, IL-6, IL-10, TNF-α and TNF-β. The ICCs were harvested 3 days post 9.0 Gy TBI. As shown in Figure 5, treatment of XBJ attenuated the expression of IL-6 (28.97 ± 0.617) and TNF-α (12.17 ± 0.498) compared with IR group (35.57 ± 0.296, 182.6 ± 1.660, respectively, p < 0.005). The expression of IL-1 (2.39 ± 0.084), IL-10 (1.18 ± 0.110) and TNF-β (3.69 ± 0.192) mRNA was increased in the XBJ-treated group compared to the IR group (0.95 ± 0.084, 0.63 ± 0.051, 0.60 ± 0.032, respectively, p < 0.01) after TBI. Thus, these results indicated that XBJ protected the small intestine against IR-induced intestinal injury by relieving inflammation.

3.6 | XBJ attenuates radiation-induced DNA damage and p53 expression in small intestine following IR treatment

To analyze whether XBJ could reduce IR-induced DNA damage, the expression of γH2AX, which is a marker that is widely used in
DNA double-strand breaks, was determined. As shown in Figure 6A, the expression of γH2AX in the small intestine tissue of the IR mice increased ([20.67 ± 3.180]/field) compared with control mice ([7.67 ± 1.453]/field, p < 0.05). XBJ treatment decreased H2AX phosphorylation ([10.33 ± 1.856]/field) compared with IR mice after 9.0 Gy IR (p < 0.05). The result indicated that XBJ could alleviate IR-induced DNA damage in the small intestine. To investigate the molecular mechanism of the protective effect of XBJ against the RIIIs, the expression of p53 was analyzed by IF analysis (Figure 6C). Compared with the control group ([4.33 ± 0.882]/field, p < 0.005), radiation caused an increase in the expression of p53 ([22.33 ± 0.333]/field) in the small intestine. In the contrast, XBJ downregulated the expression of p53 ([5.33 ± 0.882]/field, p < 0.005, Figure 6D). We also compared the expression of Bax in the ICCs (Figure 6E). Compared to the IR group, XBJ reduced the expression of Bax. To further study the radiation protection effect of XBJ, we detected the effect of XBJ on bone marrow micronucleus 3 days after 9.0 Gy TBI. As shown in Figure 6F, compared to the IR group ([186.3 ± 70.62]/1000 cells), XBJ significantly decreased radiation-induced bone marrow micronucleus formation ([20.75 ± 5.721]/1000 cells, p < 0.05). Overall, we can conclude that XBJ protects the small intestine against RII partly via the p53 pathway.

4 | DISCUSSION

High doses of IR cause massive cell death and severe damage to the GI tract referred to as GI syndrome. GI syndrome is the most common and severe complication of patients receiving radiotherapy or chemotherapy, but the cellular and molecular mechanisms
are unclear and there is no effective therapy. A previous study has shown that XBJ injection mitigates the TBI induced injury to hematopoietic cells by decreasing ROS. In this study, we have clarified the protective effect of XBJ on RIII in mice and explored its mechanism in depth.

Survival rate is a gold standard indicator of whether a drug is radioprotective. The mortality and survival days are influenced by many factors such as radiation sources, dose, and age and weight of the mice. Younger mice are more sensitive to radiation and more likely to die after irradiation. Firstly, we observed the effect of XBJ on the survival rate of mice under a lethal dose IR at an overall level that induced intestine injury. Our results showed XBJ extended the mean survival time of mice after 8.0 and 9.0 Gy TBI. The median survival of 6- to 8-week-old mice in the IR group was 5.3 days at 8.0 Gy and 4.9 days at 9.0 Gy. It should be noted that Gu et al used X-ray irradiation while we used gamma ray irradiation in this study. The intestinal epithelium is one of the fastest self-renewing tissues, and can be continuously replaced by ISCs located in the crypts. Depletion of ISCs is one of the main causes of RIII. As the apical cells fall off and ISCs died or the cell cycle is arrested, the crypts are gradually denuded and the length of the villi become shorter, leading to loss of integrity of epithelial structure and the destruction of epithelial cell homeostasis. We found that XBJ injection protected the crypt-villus structure, and increased the crypt cells and villus length after IR. ISCs are usually assessed by the expression of Lgr5. After XBJ treatment, more Lgr5+ ISCs could differentiate into villi cells and the number of Paneth cells was increased. Similarly, expression of the proliferative marker Ki67 was also increased in the intestinal crypt after XBJ treatment. Thus, XBJ may protect against RIII by enhancing the proliferation and differentiation of ISCs.

IR can cause inflammation in the body and eliminate damaged cells by recruiting neutrophils and macrophages. Tregs and their key cytokine IL-10 can promote the regeneration of stem cells, but Th1 cells and their cytokine IFN-γ suppress stem cell renewal and promote specific differentiation into Paneth cells. TNF-α acts locally in the intestinal mucosa of enteritis in an autocrine and paracrine manner. The combined action of TNF-α and IFN-γ can change the barrier function and morphology of intestinal epithelial cells, thereby increasing the permeability of intestinal mucosa and blood vessel walls, and ultimately destroying the integrity of intestinal mucosa and forming ulcers. IL-6 can induce the anti-apoptotic factors Bcl-2 and Bcl-xL through transcription activator 3 to cause abnormal accumulation of local T cells, leading to the uncontrolled secretion and adverse effect of inflammatory cytokines in the local intestinal mucosa, thereby aggravating damage to the organization of intestinal mucosa, causing the occurrence, development and persistence of enteritis. Here, we analyzed the expression of cytokines in the small intestinal crypts. Our results showed that the IL-1α, IL-6, IL-10 levels were elevated and the TNF-β levels were decreased in the ICCs of IR mice. The elevation of IL-1α and IL-6 were shown to be inhibited by XBJ. These data indicate that XBJ injection may modulate the radiation-induced inflammation.

**FIGURE 5** XBJ affects the expression of cytokines in the small intestinal crypt after TBI. The mRNA levels of IL-6 (A), IL-10 (B), IL-1 (C), TNF-α (D), and TNF-β (E) in control mice, IR mice and IR+XBJ mice were determined 3.5 days post 9.0 Gy TBI with real-time PCR. n = 5 mice per group. ***p < 0.005, **p < 0.01, *p < 0.05.
Regulating the occurrence and development of apoptosis is crucial in a variety of physiological and pathological changes caused by IR. High-dose IR causes serious DNA and protein damages in small intestinal cells, which can lead to RIII, and activate the p53 pathway. Activation of p53 regulates the cell cycle, DNA damage and repair and apoptosis. In this study, we investigated the role of the p53-dependent apoptosis pathway in the intestinal injuries sustained after IR. Previous studies reported that epithelial cell apoptosis was reduced in Bax<sup>−/−</sup> mice exposed to IR. We found that XBJ decreased the level of the expression of Bax and reduced caspase-8 and caspase-9 expression. We also found that expression of γH2AX and p53 declined after XBJ treatment. The above results indicated that XBJ alleviated IR-induced DNA damage and RIII via the p53-mediated apoptosis pathway.

In conclusion, our present studies demonstrated that XBJ injection has potential radioprotective effects against RIII in mice. XBJ attenuated IR-induced intestinal damage via the p53 pathway and relieved inflammation. These data indicated that XBJ may be a promising therapeutic candidate for relieving RIII.

**AUTHOR CONTRIBUTIONS**

Deguan Li and Aimin Meng conceived of and designed the experiment. Yinping Dong, Lu Lu and Junling Zhang carried out the experiments, analyzed the data, interpreted the results. Yinping Dong and Yuanyang Zhang contributed to data collection and interpretation. Yinping Dong, Xinyue Wang, Wenzhuang Li and Deguan Li contributed to data analysis. Yinping Dong, Deguan Li, Hui Dong, Saijun Fan contributed to manuscript preparation.
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