Polysaccharides extracted from *Rheum tanguticum* ameliorate radiation-induced enteritis via activation of Nrf2/HO-1

Tian Zhang¹,†, Lei Shi¹,†, Yan Li⁴, Wei Mu¹, HaoMeng Zhang³, Yang Li¹, XiaoYan Wang⁵, WeiHe Zhao², YuHong Qi²,* and Linna Liu¹,*

¹Department of Pharmacy, The Second Affiliated Hospital of Air Force Medical University, 710038, China
²Department of Radiotherapy, The Second Affiliated Hospital of Air Force Medical University, 710038, China
³Department of Thyroid & Breast, The Affiliated Hospital of Northwest University·XI’AN NO.3 Hospital, 710038, China
⁴Xi’an beilin Pharmaceutical Co., LTD, 710038, China
⁵Department of Rheumatology and Immunology, The Second Affiliated Hospital of Harbin Medical University, 150001, China

*Corresponding author. Department of Pharmacy, The Second Affiliated Hospital of Air Force Medical University, 710038, China. Email: linnaliuxia@163.com
†These authors contributed equally to this work.

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ABSTRACT

Radiation-induced enteritis is a major side effect in cancer patients undergoing abdominopelvic radiotherapy. The Nrf2/HO-1 pathway is a critical endogenous antioxidant stress pathway, but its precise role in radiation-induced enteritis remains to be clarified. Polysaccharides extracted from *Rheum tanguticum* (RTP) can protect the intestinal cells from radiation-induced damage, but the underlying mechanism is unknown. SD rats and IEC-6 cells were exposed to 12 or 10 Gy X-ray radiation. Rat survival, and histopathological and immunohistochemical profiles were analyzed at different time points. Indicators of oxidative stress and inflammatory response were also assessed. Cell viability, apoptosis and Nrf2/HO-1 expression were evaluated at multiple time points. Significant changes were observed in the physiological and biochemical indexes of rats after radiation, accompanied by significant oxidative stress response. The mRNA and protein expression of Nrf2 peaked at 12 h after irradiation, and HO-1 expression peaked at 48 h after irradiation. RTP administration reduced radiation-induced intestinal damage, upregulated Nrf2/HO-1, improved physiological indexes, significantly decreased apoptosis and inflammatory factors, and upregulated HO-1, particularly at 48 h after irradiation. In conclusion, Nrf2 is activated in the early stage of radiation-induced intestinal injury and plays a protective role. RTP significantly ameliorates radiation-induced intestinal injury via the regulation of Nrf2 and its downstream protein HO-1.

Keywords: Radiation-induced enteritis; Nrf2; HO-1; polysaccharides

INTRODUCTION

Radiation-induced enteritis is a major side effect in cancer patients undergoing abdominopelvic radiotherapy. Radiation therapy is an effective treatment for cancer but is associated with side effects caused by the exposure of healthy tissues adjacent to the radiation field [1, 2]. The gastrointestinal tract, especially the small intestine, is particularly sensitive to radiation, which renders it vulnerable to collateral radiation during radiotherapy for abdominal and pelvic cancers [3]. Radiation-induced enteritis and/or intestinal fibrosis occur in ~5–20% of patients treated with abdominal and pelvic radiotherapy [4, 5]. Furthermore, a considerable number of these patients require surgical intervention and/or long-term total parenteral nutrition for complications such as perforation, fistula, malabsorption or stricture, which greatly reduces their quality of life and increases the economic burden on the patients and their families [6, 7]. Radiation-induced gastrointestinal injury is histopathologically characterized by crypt cell destruction, decreased villous height and number, and impaired epithelial barrier function [8]. However, the mechanisms underlying...
the initiation and progression of radiation-induced intestinal injury remain largely unexplored [9]. There is an urgent need for the development of effective and standardized radioprotective regimens to minimize the damage caused by radiation in cancer patients with radiation-induced enteritis [10].

The transcription factor, Nrf2, which is also known as heme-binding protein 1 (HEBP1), is a potent transcriptional activator and plays a central role in the expression of many cytoprotective genes in response to oxidative and electrophilic stresses [11]. Reactive oxygen species (ROS)—induced by stress—play a pivotal role in radiation enteritis (RE). As the most important endogenous defence against oxidative stress, the Nrf2/ARE signal pathway has demonstrated a critical role in prevention and treatment of oxidative stress-induced diseases. Research has shown that Nrf2 contributes to a pro-survival response via the enhanced detoxification of pathogenic superoxides, promotion of •OH-mediated DNA damage repair and inhibition of inflammatory cytokines [12–16]. The transcription factor Nrf2 is an important modulator of antioxidant and drug metabolism, carbohydrate and lipid metabolism, as well as heme and iron metabolism [17]. As such, Nrf2 activation is beneficial for the protection of the skin from the harmful effects of ultra violet radiation (UVR) [18, 19]. Existing studies have shown that γ-ray irradiation can significantly increase the occurrence of radiation intestinal damage in Nrf2 knockout mice [20]. However, the precise role of Nrf2 in radiation-induced enteritis and its correlation with the development of disease have not been investigated, especially for X-rays [2, 21]. In addition, its role and mechanism in radiation-induced intestinal injury remain to be clarified. Therefore, this research will focus on the relationship between Nrf2/ARE pathway and RTP, through multi-period dynamic analysis of nrfl change trend, to clarify its regulatory mechanism in RE.

*Rheum tanguticum* Maxim. ex Reg. is a plant that is widely used in the treatment of digestive diseases in traditional Chinese medicine. *Rheum tanguticum* was identified by Professor Ren Yi in Northwest University in Xi’an, China, the relevant research on RTP is extracted from rhizomes, which was extracted according to the methods described previously [22, 23]. Polysaccharides extracted from *R. tanguticum* (RTP) consist of galactose, galacturonic acid, arabinose, glucose, glucuronic acid and sorbose [24]. Water-soluble polysaccharides with five different molecular weight fractions (RTP1–RTP5) have been extracted from *R. tanguticum* and separated by gel column chromatography [22]. Our preliminary animal experiments have shown that RTP1 (molecular weight, 6 × 10^5–8 × 10^5) exhibits considerable protective effects against inflammation and ulceration in rats with 2,4,6-trinitrobenzene sulfonic acid-induced colitis [25]. Nrf2 plays an important role in the body’s anti-oxidative stress response, and RTP1 also significantly inhibits cell death, reduces intracellular ROS formation and partially inhibits apoptosis after radiation-induced intestinal mucosal injury [26, 27]. However, in radiation intestinal injury, the regulatory mechanism of dynamic changes in Nrf2 during radiation-induced enteritis have not yet been studied. Also, whether RTP induces gut protection through activation of the Nrf2-ARE pathway remains to be clarified. Therefore, the purpose of the present study was to explore the dynamic changes in Nrf2 as well as the effects of RTP on these dynamic changes in response to radiation-induced bowel injury, and to identify their potential mechanism of action.

**MATERIALS AND METHODS**

**Materials**

Anti-Nrf2 antibody (ab31163), anti-heme oxygenase 1 antibody (ab13243), anti-GAPDH antibody ([EPR16891] ab181602), anti-beta tubulin antibody ([EPR1774] ab179513), anti-histone H3 antibody ([EPR17785] ab201456), anti-caspase-3 antibody (ab13847) and anti-Ki67 antibody (ab15580) were purchased from abcam (UK). Other materials were obtained from the following sources: cell counting Kit-8 (DOJINDO, Japan); SX All-In-One RT MasterMix (with AccuRT Genomic DNA Removal Kit) (abm, CAT.NO. G492, Applied Biological Materials Inc); qPCR Primer: Nrf2 (cat# RQP051369), GAPDH (cat# RQP049537), HO-1 (cat# RQP048916) were purchased from GeneCopoeia. Inc. (Shanghai, China); BCA protein assay kit (P0012S), reactive oxygen species (ROS, S0033), Hoechst33342 (C1018); cellular glutathione peroxidase assay kit (GSH, S0056), total superoxide dismutase assay kit with WST-8 (SOD, S0101), lipid peroxidation MDA assay kit (MDA, S0131), Annexin V-FITC apoptosis detection kit (Annexin V-FITC, C1063), tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6) and Interleukin-1β (IL-1β) were purchased from Beyotime Biotech Co. (Shanghai, China); nuclear and cytoplasmic extraction kit (CW0199S, Beijing, China); and medical linear accelerator (CLINAC 600C/D-SN1214, Varian Medical Systems Inc., USA). RTP was obtained from the Pharmaceutical Laboratory of the Air Force Military Medical University.

**Animals and irradiation method**

Male adult Sprague–Dawley (SD) rats, weighing 180–220 g each, were provided by the Experimental Animal Center of the Air Force Military Medical University (license number: SCXX (Army) 2016–005). All experimental procedures were approved by the Animal Experiment Administration Committee of the Air Force Military Medical University (approval number: TDLL-201603-15, date of issue: 21 March 2016). The experimental environment was as follows: temperature, 22 ± 2°C; relative humidity, 50 ± 5%; free drinking water and standard feed; and day and night alternating lighting. The rats were adapted to the feed for 7 days before the experiment. After the adaptation period, the rats were administered intraperitoneal anesthesia with 1% sodium pentobarbital, and then subjected to 12 Gy total abdominal irradiation from a 6-MV X-ray at a dose rate of 400 MU/min in the Department of Radiotherapy, Tangdu Hospital, Air Force Military Medical University. The irradiated field extended downwards from the xiphoid process to cover the entire abdomen. The source-to-skin distance was 100 cm.

**Cells and irradiation method**

We used IEC-6 cells in this study. These cells are derived from the rat small intestinal epithelial cell line CRL-1592; cells from the 14th passage were used in this experiment. IEC-6 cells were purchased from the Basic Medical Cell Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 0.01 mg/mL insulin, at 37°C in 5% CO₂. The cells were seeded at a density of 3 × 10^5 cells in 10-cm dishes, and subjected to 10 Gy total dose irradiation per dish at a dose rate of 400 MU/min. The distance from the source to the cells was
100 cm. The cells were grown until they reached log phase before being irradiated.

**Groups and treatment**

**Experiment 1**

In this survival experiment, 60 SD rats were randomly divided into the following three groups (20 rats per group): normal control (NC) group, irradiation (IR) group, and irradiation + RTP (IR + RTP) group. The rats in the IR and IR + RTP groups were administered normal saline and RTP (500 mg/kg, ig), respectively, via oral gavage, daily from 1 day before irradiation to 7 days after irradiation (Supplementary Table 1, see online supplementary material).

**Experiment 2**

In this animal model experiment, 250 SD rats were randomly divided into 25 groups (10 rats per group) according to the intervention they received (NC group, no intervention; IR groups, 12 Gy irradiation; and IR + RTP groups, 12 Gy irradiation and RTP [500 mg/kg, ig]) and the duration of observation (1–7 days; Supplementary Table 2, see online supplementary material). The rats received normal saline (IR groups) or RTP (IR + RTP groups) via oral gavage every day from 1 day before irradiation to 7 days after irradiation. Before irradiation (0 h) and at 1, 6, 12, 24, 48, 60, 72, 84, 96, 120, 144 and 168 h after irradiation, we collected jejunal tissue samples from the rats for histopathological examination and assessment of the protein and mRNA expression of Nrf2 and HO-1. At 48 h after irradiation, we detected the levels of inflammatory factors tumor necrosis factor (NF-\(\alpha\), IL-6 and IL-1\(\beta\)) and malondialdehyde (MDA) in the jejunal tissue using the appropriate kits, and the protein expression of Nrf2, Ki67 and caspase-3 using immunohistochemistry.

**Experiment 3**

In this cell experiment, IEC-6 cells were divided into 5 groups: an NC group, an IR group and three IR + RTP groups. The cells in the IR + RTP groups were treated with different concentrations of RTP (15, 10 and 5 mg/mL) before being irradiated. At 12 h after irradiation, the following indicators were analyzed: cell viability, ROS, cell apoptosis, mRNA and protein expression, and the levels of the inflammatory factors tumor necrosis factor (NF-\(\alpha\), IL-6 and IL-1\(\beta\)) (Supplementary Table 3, see online supplementary material).

**Overall observation of animals**

From 1 day before irradiation to 7 days after irradiation, rats were observed every 12 or 24 h for the following parameters: mortality, food intake, water intake, body weight and diarrhea [28] (Supplementary Table 4, see online supplementary material).

**Histopathological and immunohistochemical examination of rat jejunal mucosa**

We examined the changes in the intestinal tract of the rats in the different groups at various time points. Samples of the small intestine were collected, cleaned of mesenteric fat, and gently flushed with ice-cold phosphate-buffered saline (PBS) to remove fecal material. To facilitate comparison, histological and biochemical analyses were consistently performed on intestinal segments taken from identical anatomical positions.

In brief, the small intestine was resected, cleaned of mesenteric fat, and gently flushed with ice-cold PBS to remove fecal material. For comparative purposes, histologic and biochemical analyses were consistently performed on intestinal segments taken from identical anatomical positions. Samples were blocked with pre-cooled 4% paraformaldehyde solution, serially dehydrated, paraffin-embedded, cut into 4–6-\(\mu\)m-thin sections, dewaxed, stained with hematoxylin and eosin, dehydrated in a gradient series, put it in xylene to make the tissue transparent, mounted in neutral resin and observed under an optical microscope. Such as using the above pathological sections for immunohistochemical analysis, we need 4–6-\(\mu\)m-thin section, dewaxed, treated with 0.3% methanol–hydrogen peroxide to inactivate endogenous peroxidase, subject to antigen repair for 10 min in a microwave oven, blocked with 1% bovine serum albumin at room temperature for 30 min, incubated overnight with the primary antibody (diluted 1:100 with PBS) at 4°C and then incubated with horseradish peroxidase-labeled secondary antibody (diluted 1:200 with PBS) for 1 h at room temperature, stained with DAB, subjected to gradient dehydration, put it in xylene to make the tissue transparent, sealed with neutral resin and observed under a light microscope to evaluate the expression of Nrf2.

**Western blotting**

Jejunal tissue specimens weighing 100 mg were blotted dry on filter paper, and their cytoplasmic and nuclear proteins were extracted, according to the instructions, provided in the nuclear and cytoplasmic protein-extraction kits. Protein concentrations were measured using the BCA protein assay kit. Approximately 20 mg of total protein was electrophoresed in 10% denaturing sodium dodecyl sulfate gel and transferred to polyvinylidene fluoride membranes, which were sequentially blocked in Tris-buffered saline containing Tween 20 and 3% bovine serum albumin for 60 min at room temperature. The membranes were then incubated overnight with the primary antibodies at 4°C, followed by incubation with horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. Band intensity was visualized using an ECL kit, and gray values were determined using the ImageJ software (National Institutes of Health).

**Real-time quantitative PCR**

Approximately 50 mg of jejunal tissue was homogenized and RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific, USA). The total RNA was treated with DNA-free reagent to remove contaminating DNA. The RNA was reverse-transcribed and the mRNA level was determined using real-time quantitative polymerase chain reaction (PCR) assays. The PCR primers used are listed in Supplementary Table 4, see online supplementary material.

**Biochemical assays of rat intestinal tissue**

Approximately 50 mg jejunal tissue was homogenized into 1% tissue homogenate, which was centrifuged at 3000 rpm for 10 min. The supernatant was tested for SOD and GSH-Px activity and MDA levels, according to the instructions of the test kits used. The levels of TNF-\(\alpha\), IL-6 and IL-1\(\beta\) were measured using enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturers’ instructions.
FITC test kit, according to the manufacturer’s instructions. To stain for π-dipiodide (PI), single staining; or Annexin V-FITC and PI (double stained with 5 μL of Annexin V-FITC [single staining], 10 μL of propidium iodide (PI), single staining; or Annexin V-FITC and PI [double staining]), incubated in the dark at room temperature (20–25°C) for 10–20 min and tested using a flow cytometer.

CCK-8 assay for cell viability
IEC-6 cells in the logarithmic growth phase were seeded at a density of 5 × 10^4 cells (200 μL) per well in a 96-well culture plate. After 24 h of incubation, the cells were processed as follows: addition of 10 μL of CCK-8 detection reagent to each well, incubation at 37°C for 30 min, and measurement of the absorbance of each well with a microplate reader at a wavelength of 570 nm (each group had six replicates).

ROS assay and cell apoptosis assay
IEC-6 cells were subjected to an ROS assay using an ROS test kit, and a cell apoptosis assay using the Hoechst 33258 test kit and Annexin V-FITC test kit, according to the manufacturers’ instructions.

ROS assay
Cells in the logarithmic growth phase were seeded in 24-well culture plates at a density of 2x10^4 cells per well and incubated for 24 h, according to the instructions provided in the ROS test kit. We used the fluorescent probe DCFH-DA to detect ROS. The ROS in the cell then oxidizes the non-fluorescent DCFH to produce fluorescent DCF. DCF fluorescence indicates the level of ROS in the cell. An in situ loading probe was added, and the cells were incubated for 20 min at 37°C in an incubator. The cell samples were excited at a wavelength of 525 nm and observed under a fluorescence microscope.

Hoechst 33258 cell apoptosis assay
Cells in the logarithmic growth phase were seeded in 24-well culture plates at a density of 2x10^4 cells per well and incubated for 24 h, according to the instructions provided in the Hoechst 33258 test kit. After the addition of 200 μL of Hoechst 33258 staining solution to the cells, they were left to stand for 3–5 min at room temperature and then observed under a fluorescence microscope.

Cell apoptosis assay with Annexin V-FITC
Cells were seeded in T25 culture bottles at a density of 5 x 10^4 cells per bottle and digested with 0.25% trypsin without EDTA when they reached the logarithmic growth phase. The cells were then collected in 15-ml centrifuge tubes and centrifuged at 1000 g for 5 min. The supernatant was discarded. According to the Annexin V-FITC test kit instructions, we next added 195 μL of Annexin V-Fluorescein Isothiocyanate binding reagent and gently resuspended the cells. Cells were stained with 5 μL of Annexin V-FITC (single staining), 10 μL of propidium iodide (PI), single staining; or Annexin V-FITC and PI (double staining), incubated in the dark at room temperature (20–25°C) for 10–20 min and tested using a flow cytometer.

Immunofluorescence in IEC-6 cells
Before the experiment, 50–100 μL of membrane-breaking fluid was added to the cells for 20 min at room temperature, and this was then blocked with 3% bovine serum albumin for 30 min at room temperature. Subsequently, the cells were incubated overnight with the primary antibody to Nrf2 (diluted 1:100 with PBS) at 4°C, followed by incubation with the secondary antibody (diluted 1:200 with PBS) for 50 min at room temperature. The cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) solution for 10 min at room temperature and observed under a fluorescence microscope.

**RESULTS**

**General observation**
Compared with the NC group rats, the IR group animals exhibited apathy, reduced food and water intake, weight loss and diarrhea, including bloody diarrhea. Animals pretreated with RTP exhibited improvements, in terms of the onset, frequency and severity of symptoms. RTP improved the survival index of rats after irradiation (Fig. 1A–F).

We assessed the survival of SD rats exposed to 12 Gy total abdominal irradiation (Fig. 1A). No rats died in the NC group. In the IR group, deaths began to occur within 24 h after irradiation, and the mortality rate increased from 24 to 108 h after irradiation. At 7 days after irradiation, the survival rate was only 20% in the IR group. Among the rats that received pretreatment with RTP, the mortality rate was lower than that among the rats that received irradiation alone. In the IR + RTP group, deaths occurred between 60 and 96 h after irradiation, and at 7 days after irradiation the survival rate was 70%.

We observed the dynamic changes in the food intake of the rats before and after irradiation (Fig. 1C). The NC group rats showed stable daily food intake, but the rats in the IR group showed significantly decreased food intake after irradiation. The food intake is the lowest on the fourth day after irradiation. The rats pretreated with RTP showed significantly increased food intake compared to the rats that received irradiation alone. Water consumption also differed before and after irradiation. Compared with the NC group, the IR group and IR + RTP groups showed significantly increased water consumption 1 day after irradiation. Thereafter, the water consumption increased in the IR group and significantly improved in the IR + RTP group as compared with the IR group (Fig. 1D). The average body weight of the rats increased steadily in the NC group. In the IR group, the average body weight significantly increased slightly within 36 h after irradiation, began to decrease after 48 h, and was lowest at 96 h. The IR + RTP group rats did not exhibit any decrease in average body weight (Fig. 1B). Diarrhea scores were determined before irradiation and every 12 h after irradiation (Fig. 1E). In the IR group rats, diarrhea symptoms appeared at 60 h after irradiation, were most severe at 96 h, and disappeared after 156 h (Fig. 1F). The duration of diarrhea was 108 h. Both diarrhea scores and diarrhea duration were lower in the IR + RTP group than in the IR group.

**Microscopic changes in rat intestines**
On histopathological examination (Fig. 2), the intestinal mucosa before irradiation (0 h) showed erect, long villi; neatly arranged, compact, well-defined cells with intact surface and mucosal structures;
Fig. 1. RTP significantly improved the survival index of rats after radiation. (A) Kaplan–Meier survival curve. (B) Dynamic changes in rat body weight. (C) Dynamic changes in food intake before and after irradiation. (D) Dynamic changes in water intake before and after irradiation. (E) Changes in diarrhea scores before and after irradiation. (F) Diarrhea standard schematic (the numbers indicate diarrhea scores, from 0 to 3 points). *P < 0.05.

long and numerous intestinal glands. The number of villi decreased 12 h after irradiation in the IR group. The most severe changes were observed after 48–96 h. Intestinal injury gradually recovered 120 h after radiation, and included shortening and disappearance of the intestinal villi (observed after 48 and 60 h), severe congestion and swelling of the intestinal mucosa, short or absent intestinal glands, and thickening of the muscular layer of the intestinal wall. In the IR + RTP group, the damage was milder than that in the IR group, and the damage duration was shorter (observed after 72–84 h). Intestinal injury gradually recovered 96 h after radiation. The intestinal glands did not disappear and the intestinal villi were longer than those in the IR group. The damage to the intestinal mucosa was also milder.
Polysaccharides extracted from Rheum tanguticum

Fig. 2. Intestinal changes in rats in the IR and IR + RTP groups. The microscopic changes in the rat intestine before (0 h) and after (1–168 h) irradiation are shown. Hematoxylin and eosin staining (×100). Yellow double arrows indicate the thickness of the mucosa, congestion and swelling, and the red boxes indicates intestinal glands.

Dynamic changes in mRNA and protein expression in the jejunum

Nrf2 is an important antioxidant protein. In the IR group, Nrf2 mRNA expression slowly increased after irradiation and peaked at 12 h after irradiation. The increase in Nrf2 mRNA expression was significantly higher in the IR + RTP group than in the IR group at all time points, particularly at 12 h after exposure to RTP (Fig. 3F). HO-1 mRNA expression also slowly increased after irradiation and peaked at 48 h after irradiation. The trend of the changes in HO-1 mRNA expression was identical to that observed for Nrf2 after exposure to RTP (Fig. 3G).

Nrf2 protein expression in the cell nuclei increased slowly after irradiation, peaked at 48 h and then gradually decreased (Fig. 3A). The expression of Nrf2 protein in the cytosol also increased slowly until 12 h and then remained stable (Fig. 3B). Nrf2 protein expression was significantly higher in the IR + RTP group than in the IR group at all time points, and the trend of the changes was the same in both groups.

Oxidative stress, inflammation and immunohistochemical changes in rat tissue

Oxidative stress is one of the key mechanisms of radiation-induced injury (Fig. 4). Compared with the NC group, the IR group showed significantly reduced SOD and GSH activity and significantly increased MDA levels in tissue after irradiation. Pretreatment with RTP significantly increased the SOD and GSH activity and decreased MDA levels, thereby protecting the intestine from oxidative stress. Thus, RTP improved the biochemical indexes of oxidative stress after irradiation in rats.

RTP also enhanced Nrf2 expression and inhibited cell apoptosis (Fig. 4D). We performed immunohistochemical analyses of Nrf2, Ki-67 and caspase-3 expression in the jejunum. Compared with the NC group, the IR group showed similar Nrf2 expression and the IR + RTP group showed significantly increased Nrf2 expression at 48 h after irradiation. Ki-67 and caspase-3 expression increased after irradiation, but this increase was reduced after pretreatment with RTP.

Cell viability and apoptosis in IEC-6 cells

RTP increased the viability of irradiated cells and reduced cell apoptosis (Fig. 5). Exposure of IEC-6 cells to irradiation significantly reduced cell viability. Pretreatment with RTP significantly improved cell viability (Fig. 5A).

Cell apoptosis was detected using Hoechst 33258 (Fig. 5B) and Annexin V-FITC (Fig. 5C). The results showed that early and late apoptotic cells and dead cells were increased in irradiated IEC-6 cells. Pretreatment with RTP decreased the number of apoptotic and dead cells in a dose-dependent manner.

ROS, inflammation and Nrf2/OH-1 pathway in IEC-6 cells

Radiation enhanced the mRNA and protein expressions of Nrf2 and HO-1 in IEC-6 cells. Nrf2 and HO-1 protein and mRNA
Fig. 3. Dynamic changes in the expression of proteins and mRNA in the jejunum. (A) Western blotting showing dynamic changes in the expression of Nrf2 protein in cell nuclei. (B) Western blotting showing dynamic changes in the expression of Nrf2 and HO-1 proteins in the cytosol. Gray value calculation for (C) Nrf2 protein in cell nuclei, (D) Nrf2 protein in the cytosol, (E) HO-1 protein in the cytosol, (F) Nrf2 mRNA and (G) HO-1 mRNA. *P < 0.05.
Polysaccharides extracted from *Rheum tanguticum*

**Fig. 4.** Biochemical indexes of oxidative stress and immunohistochemical analysis in rats. Determination of (A) MDA, (B) SOD and (C) GSH content. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant. (D) Immunohistochemical analysis of Nrf2, Ki-67 and caspase-3. Blue arrows indicate typical positive cells.

Expression was significantly higher in the IR + RTP group than in the IR group (Fig. 6H). Immunofluorescence showed that RTP promoted Nrf2 entry into the nucleus. Nuclear Nrf2 expression was significantly increased after pretreatment with RTP (Fig. 6E and I). ROS, which are the main mediators of radiation-induced damage, can induce cell apoptosis and stimulate numerous inflammatory factors. ROS generation (Fig. 6F) and NF-κB and caspase-3 protein expression (Fig. 6A and B) were significantly higher in the IR group than in the NC group and significantly lower in the IR + RTP group than in the IR group. RTP also reduced the radiation-induced increase in inflammatory factors. TNF-α, IL-6 and IL-1β levels were significantly higher in the IR group than in the NC group and significantly lower in the IR + RTP group than in the IR group (Fig. 6G).

**DISCUSSION**

*Rheum tanguticum* is a traditional Chinese medicine, which is widely used in the treatment of digestive diseases [29, 30]. RTP is one of its main active ingredients. The present study showed that RTP has low toxicity and is highly safe in humans [31–35]. Radiation leads to the generation of large amounts of ROS in the body [36]. The Nrf2-ARE pathway is the most important and critical endogenous antioxidant stress pathway discovered thus far [37]. Our results showed that the Nrf2-ARE pathway plays an important protective role in radiation-induced enteritis. The results of this study also indicated that radiation can activate Nrf2 and stimulate the expression of its downstream antioxidant protein HO-1. RTP is known to be a radio-protector and can help lower the severity of radiation-induced damage at the zero
Fig. 5. RTP can increase the viability of irradiated cells and reduce apoptosis in IEC-6 cells. (A) CCK-8 test for cell viability (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant). (B) Hoechst tests for cell apoptosis (white arrows indicate apoptotic bodies). (C) Annexin V-FITC for cell apoptosis: the Q2–1 area indicates necrosis, the Q2–2 area indicates late apoptosis, the Q2–4 area indicates early apoptosis, and the Q2–3 area indicates normal cells.

time point by increasing Nrf2 expression. RTP can reduce the incidence of death and improve the physiological state of rats after radiation therapy as well as reduce the decline in cell viability, percentage of apoptotic cells and cell cycle arrest in radiation-stimulated IEC-6 cells as compared with control cells. Specifically, the protective property of RTP was observed to be related to the activation of Nrf2/HO-1 signaling.

We used an X-ray radiation rat model to investigate the regulation of Nrf2 and its downstream proteins at different time points during radiation-induced enteritis. The results showed that Nrf2 mRNA expression slowly increased after irradiation and peaked at 12 h after irradiation in the IR group. HO-1 mRNA expression also slowly increased after irradiation and peaked at 48 h after irradiation. Nuclear Nrf2 protein expression increased after irradiation, peaked at 48 h after irradiation, and then gradually reduced. Consistent with these changes, cell damage (segmental congestion of the small intestine) began to appear in the IR group rats at 24 h after irradiation. After 72–96 h, the cell damage was most severe and after 120 h the cells had started to recover. These results showed that the protective role of Nrf2 occurred in the early stages after radiation and gradually decreased after radiation.

In this study, we found that pretreatment with RTP1 significantly increased Nrf2 mRNA and protein expression as compared to the IR group at all time points. HO-1 mRNA and protein expression were also increased after pretreatment with RTP. However, the expression of Nrf2 gradually decreased in the IR + RTP group, but was still significantly higher than that in the IR group. These results showed that RTP promotes the expression of Nrf2, which increases the levels of antioxidants in the body and protects against radiation-induced injury. The corresponding ROS and cell apoptosis experiments confirmed these results. Nrf2 also participates in the regulation of the inflammatory response. NF-κB is a major transcription factor involved in inflammation-associated carcinogenesis. Proinflammatory biomarkers, such as IL-1β, IL-6, TNF-α, inducible nitric oxide synthase and cyclooxygenase-2, have been found to be induced to a greater extent in Nrf2-deficient mice than in wild-type mice [38]. Ablation of
Polysaccharides extracted from Rheum tanguticum

Fig. 6. Detection of various indicators in IEC-6 cells. (A) Western blotting showing the expression of Nrf2 and NF-κB proteins in cell nuclei. (B) Western blotting showing the expressions of Nrf2, HO-1 and caspase-3 proteins in the cytosol. Gray value calculation for (C) Nrf2 and NF-κB nuclear proteins and (D) Nrf2, HO-1 and caspase-3 cytosolic proteins. (E and I) Nrf2 immunofluorescence in cells: red fluorescence is the expression of Nrf2, blue fluorescence is the DAPI reaction to the nucleus, and the white arrows after Merge indicate the expression of Nrf2 in the nucleus. (F) ROS assay. (G) ELISA for inflammatory factors TNF-α, IL-6 and IL-10. (H) mRNA expression of Nrf2 and HO-1. *P < 0.05, **P < 0.01, ***P < 0.001.
Nrf2 seems to accelerate NF-κ B–mediated proinflammatory reactions. In this study, we found that NF-κ B expression as well as proinflammatory biomarkers, such as IL-1β, IL-6 and TNF-α, were significantly increased in irradiated IEC-6 cells. RTP exhibited a significant protective effect against radiation-induced injury in these cells, as demonstrated by the reduced expression of NF-κ B and downregulation of proinflammatory biomarkers.

The above experiments confirmed that the Nrf2/HO-1 pathway has an important protective effect in radiation intestinal injury. MAPK, PI3K, ERK, etc. are all upstream kinase pathways that can regulate Nrf2 [39, 40], and the kinase pathway participates in Nrf2. The mechanism is that protein kinase can directly phosphorylate Nrf2 to separate it from Keap1, thereby activating the Nrf2 signaling pathway [41, 42]. However, in radiation intestinal injury, the activation pathway of Nrf2/HO-1 remains to be studied. Based on this, we will in future explore the main mechanism of activation of the Nrf2/HO-1 pathway in acute intestinal injury caused by X-rays, focusing on the MAPK, PI3K and ERK pathways.

In conclusion, Nrf2 acts as an important antioxidant-regulating protein in rats. It is activated in the early stage after radiation-induced intestinal injury and plays a protective role. RTP can significantly ameliorate radiation-induced intestinal injury and may help protect against radiation-induced intestinal damage via the regulation of Nrf2 and its downstream protein HO-1.

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