Int J Clin Exp Med 2017;10(11):15196-15204
www.ijcem.com /ISSN:1940-5901/IJCEM0055809

Original Article

Protective role of enteric-coated HuDi capsules against radiation-induced intestinal injury in mice

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Received June 17, 2016; Accepted October 4, 2017; Epub November 15, 2017; Published November 30, 2017

Abstract: In this study, we investigated the protective effects of enteric-coated HuDi capsules on the intestines of irradiated mice. The abdomens of pathogen-free male C57BL/6 mice were irradiated with a Varian Clinac® 600 linear accelerator, using a 9 mV electron beam at a dose of 20 Gy. The mice were divided into 4 groups: one group was given HuDi capsules before irradiation, one group was given HuDi capsules after irradiation, one group was given amifostine before irradiation, and one group underwent radiation treatment alone. The survival rate of each group of mice was calculated, and a d-xylose absorption test was performed on the third day after irradiation. On the fourth day, the intestinal and colorectal tissues of the mice were examined for pathological changes, and immunohistochemical staining was performed to evaluate any apoptotic changes and measure the intestinal microvessel density (MVD). The group given HuDi capsules before irradiation showed the highest survival rate, the highest d-xylose absorption rate, the least pathological damage, the least vascular apoptosis, and the greatest MVD among treatment groups. Thus, these results indicate that the use of HuDi capsules protects against radiation-induced intestinal injury in mice.

Keywords: Enteric-coated HuDi capsules, radiation injury, d-xylose absorption, endothelial cell apoptosis, microvessel density

Introduction

Intestinal injury, the most common complication associated with radiotherapy for pelvic, retroperitoneal, and abdominal cavity tumors, may be caused by radiation-induced damage to the intestinal epithelial and endothelial cells and by the effects of radiation on the intestinal nervous and immune systems. Currently, there is no effective treatment available for radiation-induced intestinal injury [1]. While many abdominal organs, such as the liver and kidney, may also be affected by ionizing radiation, the small intestine is the most radiation-sensitive organ in the body. Abdominal irradiation can damage the gastrointestinal epithelial mucosa by activating inflammatory cells. The clinical manifestations of mucosal injury include destruction of crypt cells and a decrease in villi height and number [2]. Exposure to high doses of total body radiation can induce acute gastrointestinal injury, which often leads to death [3]. The high mortality is attributed to the radiation-induced damage to the intestinal and colonic mucosa [4]. It is necessary to develop novel therapeutic drugs that can prevent damage to intestinal and colonic mucosa cells. To date, most mitigating and radioprotective agents are only effective in treating the hematopoietic syndrome induced by low-dose radiation, and they fail to treat gastrointestinal injury induced by high-dose radiation.

HuDi capsules are composed of cinnabar seven, *Polygonum cuspidatum*, spreading *Hedyotis* herb, *Dahurian Patrinia* herb, garden burnet root (charcoal-treated), common *Bletilla* pseudobulb, and licorice root. Owing to their heat-clearing and blood-cooling effects, these capsules can be used to treat nonspecific ulcerative colitis and chronic bacterial dysentery. In this study, we observed that enteric-coated

HuDi capsules protect the intestinal mucosa, preserve its ability to absorb nutrients, and delay the apoptosis of the intestinal vascular endothelial cells that other works have shown to be the main targets of radiation damage [5].

Material and methods

Animal grouping and drug administration

Eighty 6-week-old male SPF C57BL/6 mice, weighing 20 ± 2 g, were purchased from the SLAC Laboratory Animal Company (Shanghai, China). All animal handling procedures were reviewed and approved by the Animal Care/User Ethical Committee of Soochow University, Suzhou City, P.R. China (approval number 20100909). After being allowed to acclimate to their conditions for a week, the mice were randomly divided into two groups, one selected for tissue sampling and the other for assessment of post-irradiation survival. Each group was further divided randomly into four groups of 10 mice each: the enteric-coated HuDi capsule pre-irradiation group (HuDi + IR), enteric-coated HuDi capsule post-irradiation group (IR + HuDi), amifostine pre-irradiation group (amifostine + IR), and control vehicle irradiation group (IR).

The enteric-coated HuDi capsules were administered at a dose of 1.0 mg/mouse by the i.g. route. The HuDi + IR group was given capsules at a dose of 1.0 mg/mouse, once daily for two consecutive days, before irradiation. The IR + HuDi group was given HuDi for two consecutive days after irradiation. Mice in the amifostine + IR group were given 15 mg/kg amifostine 30 min before irradiation, by abdominal injection. The IR group was injected with 100 μl of PBS.

Reagents and equipment used

The cleaved caspase-3 antibody was purchased from Cell Signaling Technology (Cat. No. 9664). The CD31 antibody was purchased from Abcam (Cat. No. ab28364). The citrate buffer solution (pH 6.0) was prepared by adding 0.8 g citric acid and 6 g sodium citrate to 2000 ml single-distilled water. The double staining kit (Cat. No. DS204C-6) for immunohistochemical analysis was purchased from GBI Labs (U.S.). Enteric-coated HuDi capsules were purchased from Anhui Jorfar Pharmaceutical Co., Ltd. A TUNEL kit was purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). The ultra-microtome was purchased from Austria, and the cabinet dryer was purchased from Leica Microsystems.

Irradiation conditions

Site-specific irradiation of mice was performed at the radiotherapy center of the Suzhou Kowloon Hospital with a 9 mV electron beam from a linear accelerator (Clinac® 600, Varian Medical Systems, CA, U.S.), at a source-skin distance of 100 cm. The radiation field was localized to the abdomen and the dose rate was 200 cGy/min. The group selected for tissue sampling received a dose of 10 Gy, and the group selected for assessment of post-irradiation survival received a dose of 20 Gy. All mice were anesthetized with 1% pentobarbital sodium solution (i.p., 0.15 ml/mouse) prior to irradiation.

Estimation of D-xylose absorption rate

Each mouse was administered 100 μl of 5% D-xylose solution on the third day after irradiation, and urine samples were collected after 2 h. The color reagent was then added to the urine at a ratio of 1:100 and the samples were heated for 4-5 min at 100°C and then cooled to room temperature. After zero was set at 554 nm using deionized water, the absorbance of the urine collected from mice that were not given D-xylose solution was recorded and set as the background value. Then, the absorbance values of the urine from d-xylose-administered mice were recorded at 554 nm. D-xylose concentration in urine was thus calculated from a standard curve plotted using the absorbance values of standard D-xylose solution. The absorption rate was determined for each group by dividing the value obtained from these calculations by the oral dosage taken, and comparisons were then made among the different groups.

Histopathological analysis of the small intestine

On the fourth day after irradiation, paraffin sections of tissues from the duodenum, jejunum, ileum, and colonic rectum were made for microscopic imaging at 200 × magnification, with 10 fields recorded per section.

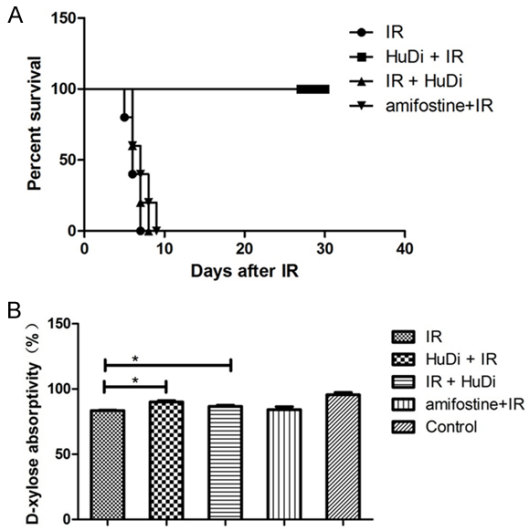

Figure 1. Effect of enteric-coated HuDi capsules on (A) survival rates and (B) D-xylose absorptivity of irradiated mice (*P<0.01).

Detection of endothelial cell apoptosis by double immunohistochemical staining and TUNEL assay

The intestinal sections were baked at 60°C for 60 min, deparaffinized with xylene, and hydrated with gradient alcohol. Endogenous peroxidases and alkaline phosphatases were blocked using small-intestine-specific peroxidase and alkaline phosphatase blockers. After performing antigen retrieval using citrate buffer solution, the sections were blocked with 10% sheep serum, incubated at room temperature for 50 min with CD31 antibody, and then stained using GBI-Permanent Red. After blocking with Blocker A and Blocker B from the reagent kit, the sections were incubated at room temperature for 50 min with cleaved caspase-3 antibody (1:800) and then stained with emerald green. Finally, the sections were mounted with a hydrophilic mounting medium, allowed to dry, and covered with a glass coverslip for microscopic observation. The value obtained on dividing the number of double-positive endothelial cells by the number of CD31-positive cells gave the percentage of apoptotic endothelial cells. All evaluations were made on 10 fields per section (400 ×).

TUNEL assay was performed according to the protocol of the In Site Cell Apoptosis Detection Kit (Lot No. 08E20B).

Microvessel density (MVD)

Immunohistochemical staining with CD31 antibody was used to visualize the blood vessels in the tissue. The stained vascular tissue was observed using light microscopy at 100 × magnification to find the five regions with the highest CD31 densities. The stained vessels in the fields were counted at 400 × magnification.

Evaluation of angiogenesis: Any positively stained endothelial cells or cell clusters that were clearly separated from the surrounding blood vessels, tumor cells, and other connective tissue components were considered as countable microvessels, regardless of the presence of a lumen.

Observation of mortality

To determine the survival rates, mice in the survival rate group were raised in a sterile chamber after being irradiated with a 20 Gy electron beam. They were observed for 30 days, and mortality was recorded daily.

Statistical analysis

Each experiment was performed 3-4 times. The Kaplan-Meier method was used to evaluate the survival rate. Other data were analyzed using the SPSS 18.0 software. Data are presented as mean ± standard deviation (SD), and differences among groups were compared using ANOVA analysis. For D-xylose absorptivity, the villi length and the crypt quantity and the apoptosis rate were assessed, and the differences between two groups (relative to the IR control group) were determined using a t-test. P<0.05 was considered statistically significant.

Results

Survival rate and D-xylose absorptivity of irradiated mice

All mice in the HuDi + IR group survived after irradiation with a 20 Gy electron beam, whereas the mice in all other groups died within a week (**Figure 1A**). In this way, pretreatment with enteric-coated HuDi capsules significantly improved the survival rate of irradiated mice.

D-xylose is usually absorbed directly by the small intestine and excreted via urine, without

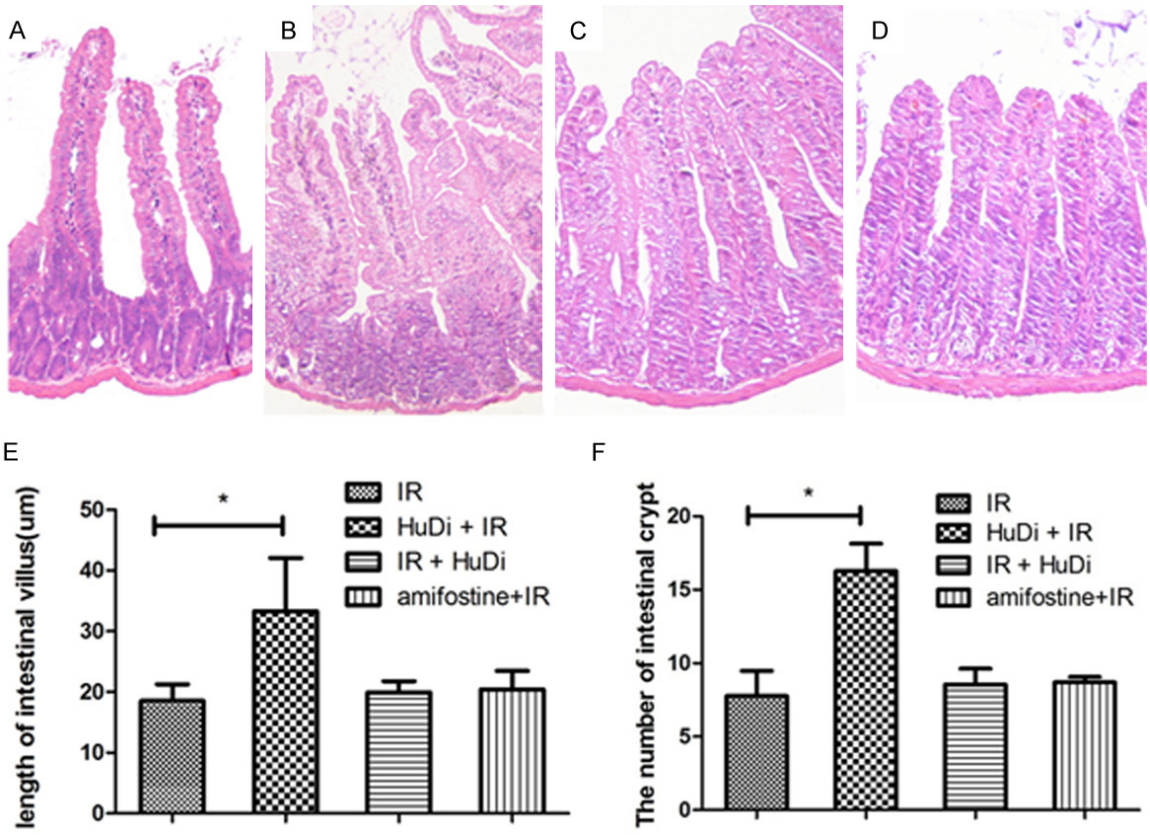

Figure 2. Histopathological analysis of the effect of enteric-coated HuDi capsules on the small intestine tissues of irradiated mice: (A) HuDi + IR group. (B) IR group. (C) IR + HuDi group. (D) Amifostine + IR group. Effect of enteric-coated HuDi capsules on intestinal villi length (E) and (F) the number of crypt cells (*P<0.05). Magnification 400 ×.

any involvement by digestive enzymes or metabolism in the liver. For these reasons, we can investigate the impact of drugs on the functioning of the small intestine by measuring the D-xylose levels in blood or urine during a certain period after drug administration.

When we measured the D-xylose absorption in mice irradiated with a 10 Gy electron beam, the highest absorption rate was observed in normal mice, and the second highest and the lowest in the HuDi + IR and IR groups, respectively (**Figure 1B**). There were statistically significant differences among the HuDi +IR, IR + HuDi, and amifostine + IR groups in terms of absorption (P<0.05).

Histopathological analysis of the small intestines of irradiated mice

The histopathological changes observed in the small intestines of mice after irradiation with a 10 Gy electron beam were as shown in **Figure 2**. The small intestine tissue sections of mice in the HuDi + IR group appeared sharper, showing arranged villi with deep-sunken cryptae and more abundant glands than in the corresponding tissue sections of mice in the IR group. Further, the structure was relatively unabridged and there was less inflammation in the HuDi + IR group than in the other groups.

While inflammation in the amifostine + IR and IR + HuDi groups was milder than in the IR group, it was more severe than that observed in the HuDi + IR group. The HuDi + IR group had statistically significantly longer villi (**Figure 2E**) and more cryptae (**Figure 2F**) than in the IR group (P<0.05).

Analysis of endothelial cell apoptosis in irradiated mice via double immunohistochemical staining and TUNEL assay

When immunohistochemical analysis of the small intestinal (**Figure 3A-D**) and colorectal

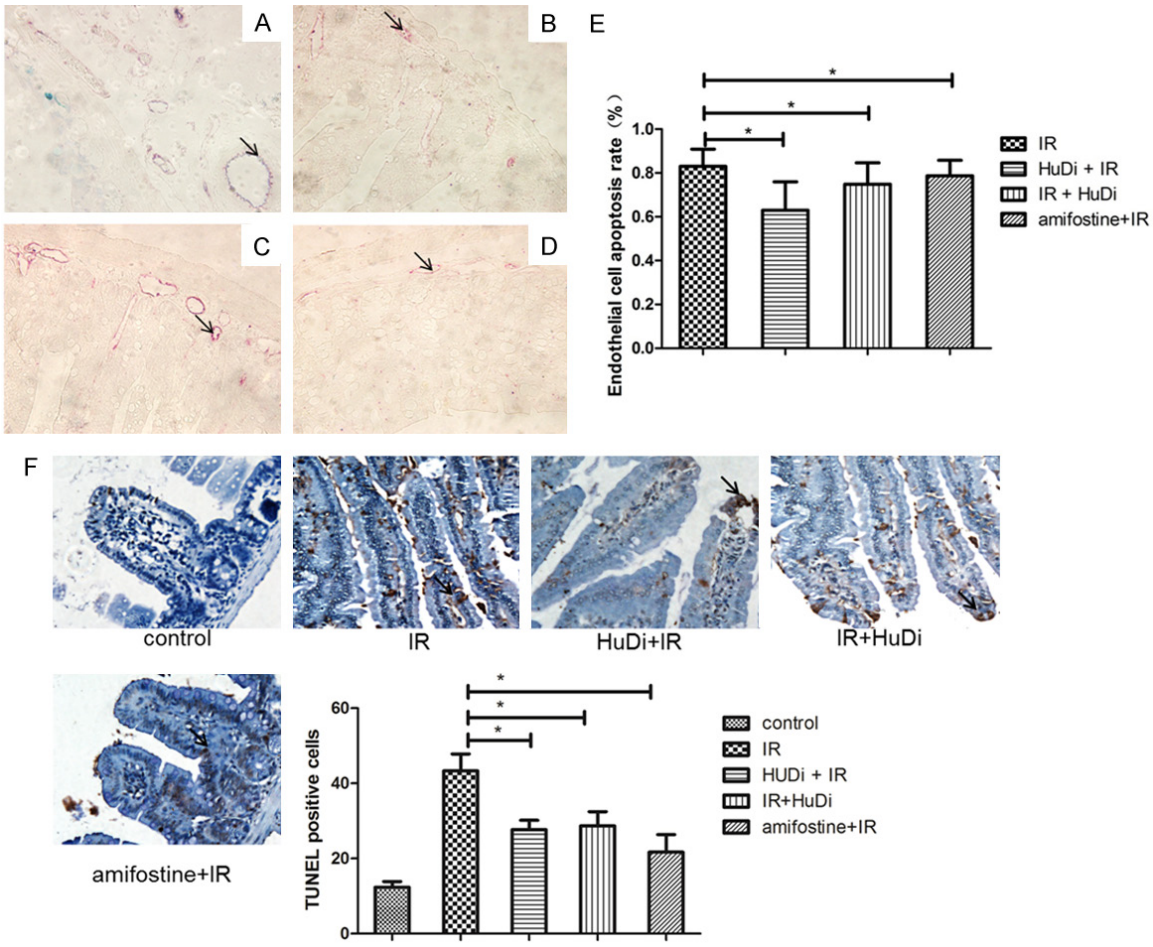

Figure 3. Analysis of the effect of enteric-coated HuDi capsules on small intestinal endothelial cell apoptosis in irradiated mice via immunohistochemical staining: A. HuDi + IR group. B. Amifostine + IR group. C. IR + HuDi group. D. IR group. E. Apoptosis rates. F. TUNEL assay. (*P<0.05). Magnification was 400 ×.

(**Figure 4A-D**) endothelial cell apoptosis was performed, CD31-positive endothelial cells were stained pink, cleaved caspase-3-positive cells were stained green, and the CD31/cleaved caspase-3 double-positive cells were stained blue-purple. When the apoptosis rates of the small intestinal (**Figure 3E**) and colorectal (**Figure 4E**) endothelial cells were quantified, the IR group mice showed the highest rates of apoptosis among all groups. TUNEL assays (**Figures 3F** and **4F**) also showed that the HuDi + IR group had a significantly lower rate of apoptosis (P<0.05) than the IR, IR + HuDi, and amifostine + IR groups.

Estimation of the MVD in irradiated mice

The effect of enteric-coated HuDi capsules on the small intestinal and colorectal MVD in irradiated mice was investigated by visualizing and counting the CD31-positive microvessels in the small intestinal and colorectal tissues via immunohistochemical staining. The small intestinal MVD was significantly higher in the HuDi + IR group than in the IR group (P<0.05) (**Figure 5A**). Similarly, the HuDi + IR group also had a significantly higher colorectal MVD than the IR group (P<0.05) (**Figure 5B**).

Discussion

Nuclear technology is currently widely used in the military, medical, scientific, industrial, and agricultural sectors. Detonation of nuclear weapons, radiation disasters, excessive radiotherapy, and total body irradiation prior to bone marrow transplantation can all cause radiation damage. Specifically, radiotherapy has become one of the most economical and efficacious therapeutic strategies for treating malignant tumors [6].

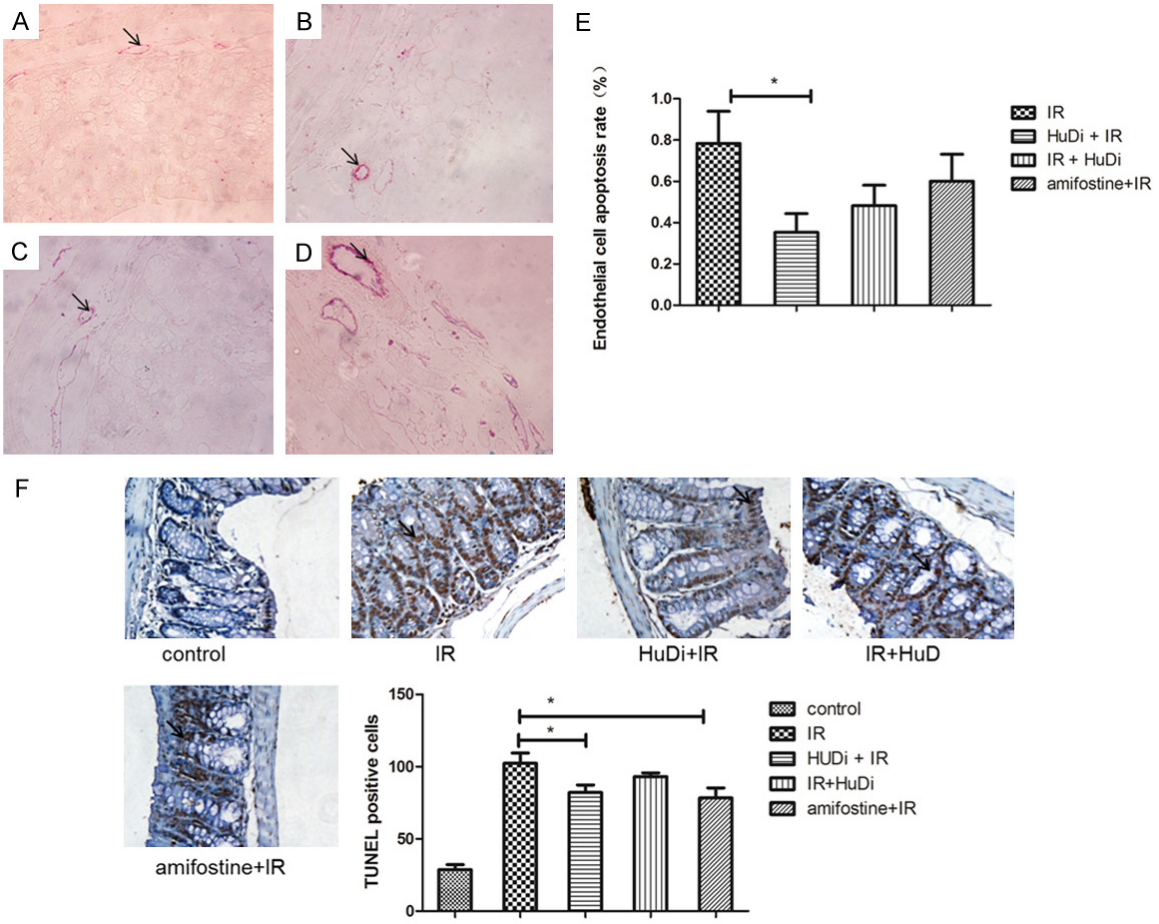

Figure 4. Analysis of the effect of enteric-coated HuDi capsules on colorectal endothelial cell apoptosis in irradiated mice via double immunohistochemical staining: A. HuDi + IR group. B. IR + HuDi group. C. Amifostine + IR group. D. IR group. E. Apoptosis rates. F. TUNEL assay. (*P<0.05). Magnification was 400 ×.

Although the clinical application of radiotherapy is becoming increasingly common, tissue damage during radiotherapy is unavoidable. Gastrointestinal injury is one of the most common complications of radiotherapy, occurring in more than 75% of the patients [7].

As one of the prime causes of intestinal mucosal barrier damage, radiation can lead to destruction of small intestinal villi, death of proliferating crypt cells, intestinal bacterial translocation, mucosal ulceration, necrosis, hemorrhage, intestinal obstruction, intestinal fistula, abscess formation, and peritonitis [8-9]. It can also cause mortality because of fatal complications such as endogenous sepsis and multi-organ failure. Because radiation-induced intestinal injury has a high incidence and mortality rate and requires expensive remedial treatment, any treatment that can protect the intestine from radiation damage, accelerate the healing of radiation-induced intestinal injury, or reduce the complication rate is beneficial [10].

This study has shown that pretreatment with enteric-coated HuDi capsules has a protective effect on the intestinal mucosa. It can also help maintain the integrity of small intestinal villi, reduce damage to crypt cells, and preserve the absorptive function of the small intestine.

The anti-radiation agents that are currently used, such as sulfur compounds, hormones, cytokines, and some herbal preparations, have their own shortcomings [11, 12]. For example, sulfur compounds exert anti-radiation effects only above a certain concentration, which often leads to toxic effects. Similarly, prolonged hormone usage affects endogenous hormone lev-

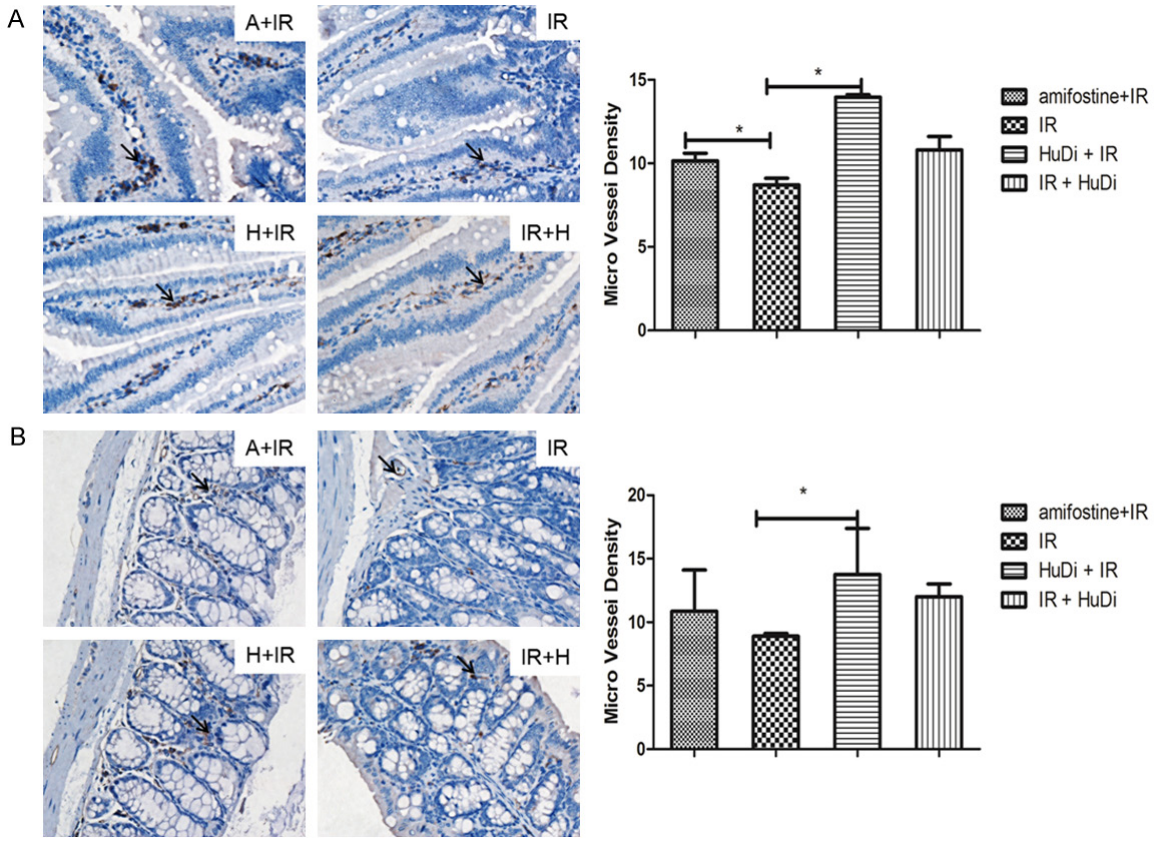

Figure 5. Immunohistochemical analysis of the effect of enteric-coated HuDi capsules on (A) small intestinal and (B) and colorectal microvessel density in irradiated mice (*P<0.05). Magnification was 400 ×.

els, resulting in serious side effects. Research has found that traditional Chinese medicines exert their anti-radiation effects mainly by protecting cells from oxidants, protecting hematopoietic tissues, improving microcirculation, enhancing immunity, promoting cell proliferation, and inhibiting cell apoptosis [13].

In enteric-coated HuDi capsules, *P. cuspidatum* can dispel flatulence and dampness and eliminate blood stasis. In addition, *P. cuspidatum* has also been shown to exert immunity-boosting and anticancer effects. Burnet carbon exerts heat-clearing, blood-cooling, hemostatic effects, sore detoxification, and sour convergence effects. *Patrinia*, which is capable of resolving heat, detoxifying, alleviating pain, promoting pus evacuation, and eliminating blood stasis, is used clinically for the treatment of abdominal pain and other symptoms. Cinnabar seven has anticonvulsant, sedative, anti-corrosive, and other activities. The capsule formulation also contains several other drugs that can restore vital energy, stop diarrhea, and dispel flatulence [14].

The results of this study suggest that enteric-coated HuDi capsules reduce radiation-induced intestinal damage by improving endothelial cell function. Radiotherapy-induced injury to intestinal capillary endothelial cells has already been reported to be the initial cause of radiation enteritis [15].

Paris et al. reported that the aggravation of radiation-induced intestinal injury in mice was prevented when endothelial cell apoptosis was inhibited by intravenous basic fibroblast growth factor (bFGF) or acid sphingomyelinase gene deletion [5]. They also found that endothelial cells expressed bFGF receptor transcripts and that crypt cells did not, which suggested that the endothelial lesions form before crypt stem cell damage takes place. However, Kirsch et al. observed that selective deletion of the pro-apoptotic *Bak1* and *Bax* genes from the gastrointestinal epithelium or from endothelial cells did not protect irradiated mice from vascular endothelial cell apoptosis or death due to gastrointestinal syndrome [16]. They found that crypt epithelial cells still died, although to a

lesser degree, which suggested that endothelial cell apoptosis plays only a moderate role in radiation-induced intestinal injury. Thus, some scholars believe that two types of cell may be involved in radiation-induced intestinal injury; crypt epithelial cells could be the most important target of radiation injury, and endothelial cell apoptosis may be a sign of radiation-induced injury to crypt epithelial cells [17].

In summary, enteric-coated HuDi capsules can improve the survival rate of mice with radiation-induced intestinal injury, protect the intestinal mucosa and the absorptive function of the small intestine, and reduce endothelial cell apoptosis. Although the specific mechanism of action needs to be investigated further, enteric-coated HuDi capsules can provide a new way forward in the treatment of radiation-induced intestinal injury.

Acknowledgements

This work was supported by the Traditional Chinese Medicine Research Project of National Health and Family Planning Commission of Anhui Province (grant number 2016zy33), the National Natural Science Foundation of China (grant number 81372920 and 81573077), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). And we thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

Disclosure of conflict of interest

None.

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