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Experimental Animal Model of Re-irradiation to Evaluate Radiation-induced Damage in the Normal Intestine

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Abstract. *Background/Aim: We aimed to elucidate the pathological findings following acute and late re-irradiation in a preclinical model. Materials and Methods: Mice were divided into five treatment groups: sham-irradiation (Sham-IR), 10-12 Gy (Single IR Acute), 15 Gy (Single IR Late), 15 Gy followed by 10-12 Gy re-irradiation 7 days later (Re-IR Acute), or 15 Gy followed by 10-12 Gy re-irradiation 12 weeks later (Re-IR Late). Mice were sacrificed after either single irradiation or re-irradiation for pathological assessment. Results: The Re-IR Late group had significantly lower numbers of crypts with apoptotic cells than those observed in mice in the Single IR Acute group. There were no significant differences between the Single IR Acute and re-IR Acute groups in cell proliferation or in a crypt survival assay. Conclusion: Re-irradiation with a long interval after the first irradiation may cause similar acute biological effects in normal intestine as observed following irradiation without re-irradiation.*

Recent advances in modern radiotherapy, including intensity-modulated radiation therapy, stereotactic body radiation therapy, and charged particle therapy, have made re-irradiation feasible for some clinical indications (1-3). Re-irradiation for recurrent and secondary primary tumors can be clinically beneficial and reasonably well tolerated (1-10). Longer survival of some patients due to the improvement in cancer treatments over time has resulted in an increased need for re-irradiation, especially in patients with loco-regionally recurrent disease who are resistant to chemotherapy. While salvage surgery is a potential treatment option for loco-regional recurrence following radiotherapy, surgical procedures may be difficult due to secondary to late radiation effects, including anatomical changes and fibrosis of normal tissue surrounding the tumor (11, 12). Re-irradiation, if tolerable, may be a better therapeutic option for these patients.

Toxicity from re-irradiation can be lethal in some situations, and the safety and feasibility of re-irradiation in many situations is not well defined and is an important area for investigation (4-7, 13-18). Previous studies have focused on re-irradiation in preclinical models (13-18), and it has been reported that the interval between the initial radiation and re-irradiation is associated with clinical outcomes and the incidence and severity of toxicities (8-10, 13). Pathological changes after irradiation of the normal intestine include damage, such as submucosal fibrosis and hypoxia, that can be observed at 12 weeks following irradiation in preclinical models (19-21). To the best of our knowledge, little work has been done in preclinical models of re-irradiation-induced gastrointestinal injury (18). The purpose of the experiments described here was to study the pathological changes that occur following re-irradiation as a function of the time interval between initial irradiation and re-irradiation in a preclinical model.

Materials and Methods

All animal experiments were approved by our Institutional Animal Use Committee (approval number: KAME-29-033).

Male C57BL/6J mice (8-weeks-old) were divided into five groups as follows: sham-irradiation (Sham-IR), 10-12 Gy (Single IR Acute), 15 Gy (Single IR Late), 15 Gy followed by 10-12 Gy re-irradiation 7 days later (Re-IR Acute), or 15 Gy followed by 10-12 Gy re-irradiation 12 weeks later (Re-IR Late). We compared the pathological findings between the groups. The grouping and schema of the experiments are shown in Table I.

Irradiation was performed at 80 kV and 1.25 mA with a dose rate of approximately 35.2-39.6 cGy/min using an X-ray unit. Mice in the Single IR Acute group received 10-12 Gy total body irradiation. For their first radiation treatment, mice in the Re-IR Acute, Single IR Late, and Re-IR Late groups received abdominal irradiation of 15 Gy in a single fraction under anesthesia. Lead shielding was used to cover the mice, excluding the whole abdomen. A peritoneal injection of a combination of medetomidine (0.3 mg/kg), butorphanol (5 mg/kg), and midazolam (4 mg/kg) was used for anesthesia. For the second irradiation (re-irradiation), mice were administered total body irradiation of 10 or 12 Gy in a single

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Table I. *Schema of the experimental model of re-irradiation.*

	Treatment			0 h	Sacrifice	
Group	1st IR (Abdominal)	Interval	2nd IR (TBI)	6 h	72 h	84 h (3.5 days)
Sham-IR	0 Gy†		0 Gy†		(Biological endpoints)	
Single IR Acute	0 Gy†		10 or 12 Gy	Apoptosis	Cell proliferation	Surviving crypts
Single IR Late	15 Gy	12 weeks	0 Gy†			
Re-IR Acute	15 Gy	1 week	10 or 12 Gy			
Re-IR Late	15 Gy	12 weeks	10 or 12 Gy			

†Sham irradiation.

fraction without anesthesia 7 days (Re-IR Acute group) or 12 weeks (Re-IR Late group) after the first irradiation. For an experiment assessing intestinal crypt epithelial cell survival and other experiments, total body irradiation of 12 Gy and 10 Gy was administered, respectively.

Mice were sacrificed for assessment of apoptosis 6 h after re-irradiation, at which time the intestines in a part of the jejunum were harvested for pathological examination. In some experiments, mice were sacrificed 72 h after re-irradiation and 1.5 h after the administration of bromodeoxyuridine (BrdU; 100 mg/kg, IP).

To assess the differences between the effect of the first and second irradiation on normal intestinal crypt epithelial cell survival, we used an established microcolony assay as previously described (22, 23). In this experiment, the number of viable crypts/cross-section in the jejunum were measured 84 h after re-irradiation.

Pathological assessment and immunohistochemistry. Harvested tissues were fixed in 4% paraformaldehyde phosphate buffer solution, and then stained with hematoxylin and eosin. Details of the pathological experiment and immunohistochemistry (IHC) were as described in previous reports (23, 24). DNA fragmentation and cell proliferation were examined histologically using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) and BrdU staining, respectively. For quantification of IHC-positive cells, tissue sections were analyzed at 40× magnification, and 25 cells from the bottom of the crypt were counted. Apoptotic cells were defined as TUNEL-positive cells. The apoptotic index was defined as the percentage of TUNEL-positive cells in each crypt. The incidence of BrdU positive cells was defined as the percentage of BrdU-positive cells in each crypt. The incidence of IHC-positive cells was analyzed in 100 crypts from 16 slices of intestine from two mice.

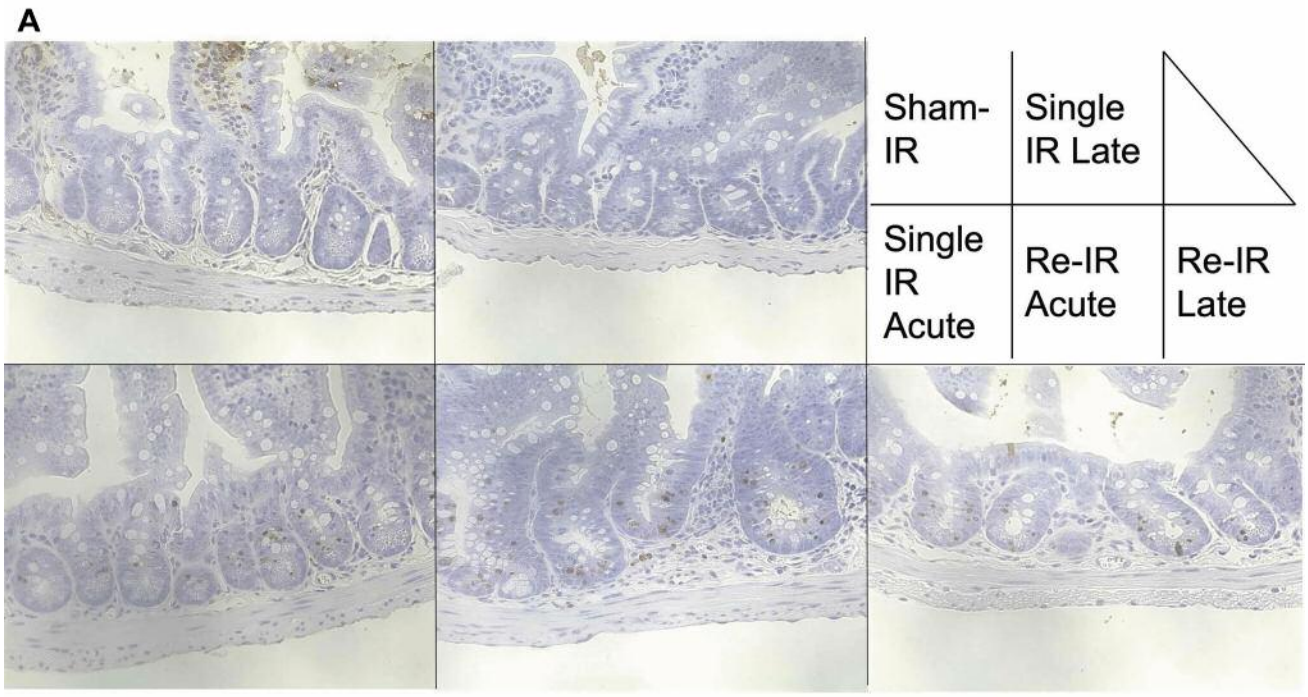
Statistical analysis. The data are expressed as the mean with standard deviations in parentheses unless otherwise indicated. Data were analyzed using a two-tailed Fisher's test or a Mann-Whitney U test. All analyses were performed using GraphPad Prism version 8.2.1 (GraphPad Software, Inc., San Diego, CA, USA) and $p<0.05$ was considered to indicate a statistically significant difference.

Results

Incidence of intestinal crypts with apoptotic cells. Sixteen intestinal sections from two mice were analyzed in each group. No significant differences were observed in the incidence of crypts with apoptotic cells between the Sham-IR and Single IR Late groups (Figure 1). The Re-IR Late group had a significantly lower incidence of crypts containing at least one apoptotic cell than the Single IR Acute group. There was no significant difference in the incidence of crypts with apoptotic cells between the Sham-IR and Single IR Late groups. Radiation exposure caused a significant increase in the incidence of apoptosis-containing crypts after both the first irradiation and re-irradiation.

Apoptotic index. We counted the apoptotic cells from 25 cells at the bottom of each crypt. Crypts with at least one apoptotic cell were assessed in the Single IR Acute, Re-IR Acute, and Re-IR Late groups (Figure 2). In each group, 100 crypts from 5-8 sections from two mice were randomly selected and analyzed. In the Sham-IR and Single IR Late groups, 18 and 17 crypts with no apoptotic cells, respectively, were included. No significant differences were observed between the Sham-IR and Single IR Late groups and the Single IR Acute and Re-IR Late groups. However, the Re-IR Acute group had a significantly higher apoptotic index than that of the Single IR Acute and Re-IR Late groups ($p<0.0001$ and $p<0.0001$, respectively; Figure 2A). Although similar apoptotic patterns were observed in the Sham-IR and the Single IR Late groups, the Re-IR Acute group had apoptotic cells extending further up the crypts than those of the Single IR Acute and the Re-IR Late groups (Figure 2B and C).

Cell proliferation after re-irradiation. To assess recovery after re-irradiation, we counted the BrdU positive cells from 25 cells from the bottom of each crypt. In each group, 100 crypts from 6-8 sections from two mice were randomly selected and analyzed. There were no significant differences in the number of BrdU positive cells in each crypt between the Single IR Acute and Re-IR Late groups (Figure 3). As the baseline, the Single IR Late group had less cell proliferation than the Sham-IR group ($p<0.0001$; Figure 3B). The Re-IR Late group had slightly lower BrdU-positive incidence than that of the Single IR Acute group, with no significant differences between the two groups. However, the Re-IR Acute group had fewer BrdU positive cells, indicating



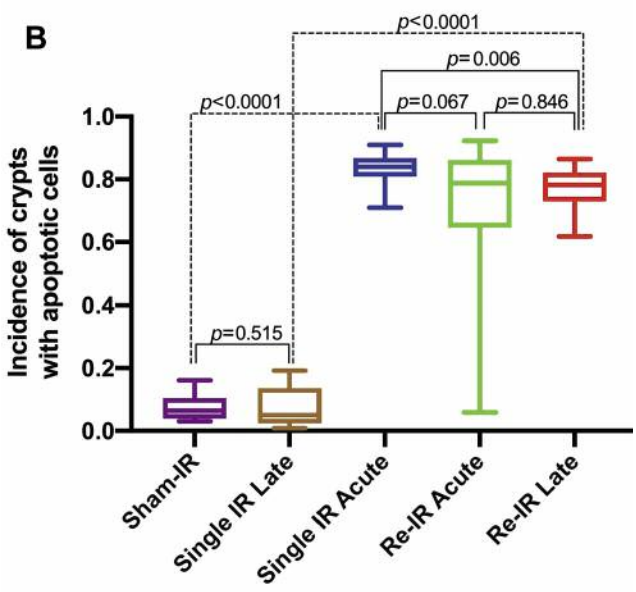


Figure 1. *Incidence of intestinal crypts with apoptotic cells. TUNEL staining reveals apoptotic cells in crypts in each group (A; TUNEL staining; original magnification, 40×). The number of crypts with at least one apoptotic cell in each group is shown (B). The number of crypts with apoptotic cells was 0.076 (0.045), 0.076 (0.060), 0.836 (0.046), 0.709 (0.227), and 0.777 (0.063) in the Sham-IR, Single IR Late, Single IR Acute, Re-IR Acute, and Re-IR Late groups, respectively.*

less cell proliferation, than the Single IR Acute group and Re-IR Late group ($p<0.0001$ and $p<0.0001$, respectively). A similar pattern of BrdU positive cells was observed between the Sham-IR and Single IR Acute groups, and between the Single IR Acute and Re-IR Late groups (Figure 3C and D). However, the Re-IR Acute group had a lower level of cell proliferation in each position of the crypts (Figure 3D).

Intestinal crypt stem cell assay. We assessed intestinal stem cell survival using an intestinal crypt assay (Figure 4). For this experiment, 16, 16, 53, 43, and 54 sections from 2, 2, 4, 3, and 4 mice were used in the Sham-IR, Single IR Late, Single IR Acute, Re-IR Acute, and Re-IR Late groups, respectively. After irradiation, intestinal stem cell survival was significantly lower in the Single IR Acute and Re-IR Late groups than in the Sham-IR and Single IR Late groups ($p<0.0001$ and $p<0.0001$), respectively. No significant differences were observed between the Sham-IR and Single IR Late groups, or among the Single IR Acute, Re-IR Acute, and Re-IR Late groups.

Finally, submucosal fibrosis was observed 12 weeks after the first irradiation in the Single IR Late group (Figure 5A and B).

Discussion

The clinical feasibility and utility of re-irradiation have been described in patients with various primary tumor sites (1-10). However, there are few reports on pathological changes in normal intestine that focus on the interval between the first and second irradiation (13-18). In the study described here, we used an experimental animal model to analyze apoptosis, cell

proliferation, and crypt survival after re-irradiation of the intestine. In this study, re-irradiation with a long interval after the first irradiation was shown to have equivalent biological effects on the normal intestine to those of an acute reaction.

An increased interval between the first irradiation and re-irradiation can ameliorate the biological responses that lead to severe toxicity, even if the cumulative physical doses of irradiation are the same (8-10, 13). For example, Simmonds *et al*. reported little or no residual injury after re-irradiation 17-52 weeks after the initial irradiation in pig dermal tissue (13). Raynaud *et al*. compared intervals from 2-12 months and reported that previously irradiated mice had greater radioresistance in the intestine than non-irradiated controls (18). Our study also demonstrated that an extended interval of 12 weeks resulted in equivalent or slightly lower radiation-induced toxicity in the normal intestinal epithelium in the Re-IR Late group compared to that observed in the Single IR Acute group.

We hypothesized that extended time intervals between radiation exposure would lead to hypoxia due to submucosal fibrosis (Figure 5) (19, 20). However, the Re-IR Acute group had more cell death and less cell proliferation than both the Single IR Acute and Re-IR Late groups. Insufficient recovery from the first irradiation seemed to cause higher levels of apoptosis and lower levels of cell proliferation, because the location of apoptotic cells shifted to higher positions in the crypts in the Re-IR Acute group that in the Single IR Acute and Re-IR Late groups. Notably, cell proliferation was lower after an extended interval from irradiation (Single IR Late group) in comparison with that of the non-irradiated control (Sham-IR group). Because these data may suggest that previously irradiated normal tissues have impaired recovery from radiation-induced damage compared to unirradiated tissue, further study regarding this delayed response after re-radiation exposure is needed.

We have shown that the interval between the first irradiation and re-irradiation is an important determinant of radiation-induced damage in normal tissues. To the best of our knowledge, there are only a few reports describing apoptosis and cell proliferation after re-irradiation. In addition, there is a lack of data from studies that directly analyze acute and extended intervals between the first irradiation and re-irradiation. The present study showed significant differences in indicators of radiation-induced damage after re-irradiation at two different time points (12 weeks *vs*. 7 days after the first irradiation).

This study had several limitations. First, only two time points (12 weeks and 7 days after the first irradiation) and a limited number of animals were included in this study. The inclusion of more time points may better define the timeline for induction/repair of radiation damage following re-irradiation. In addition, multiple sections were assessed to increase the number of reliable quantitative data points. Reynaud *et al*. have reported that the acute response to re-

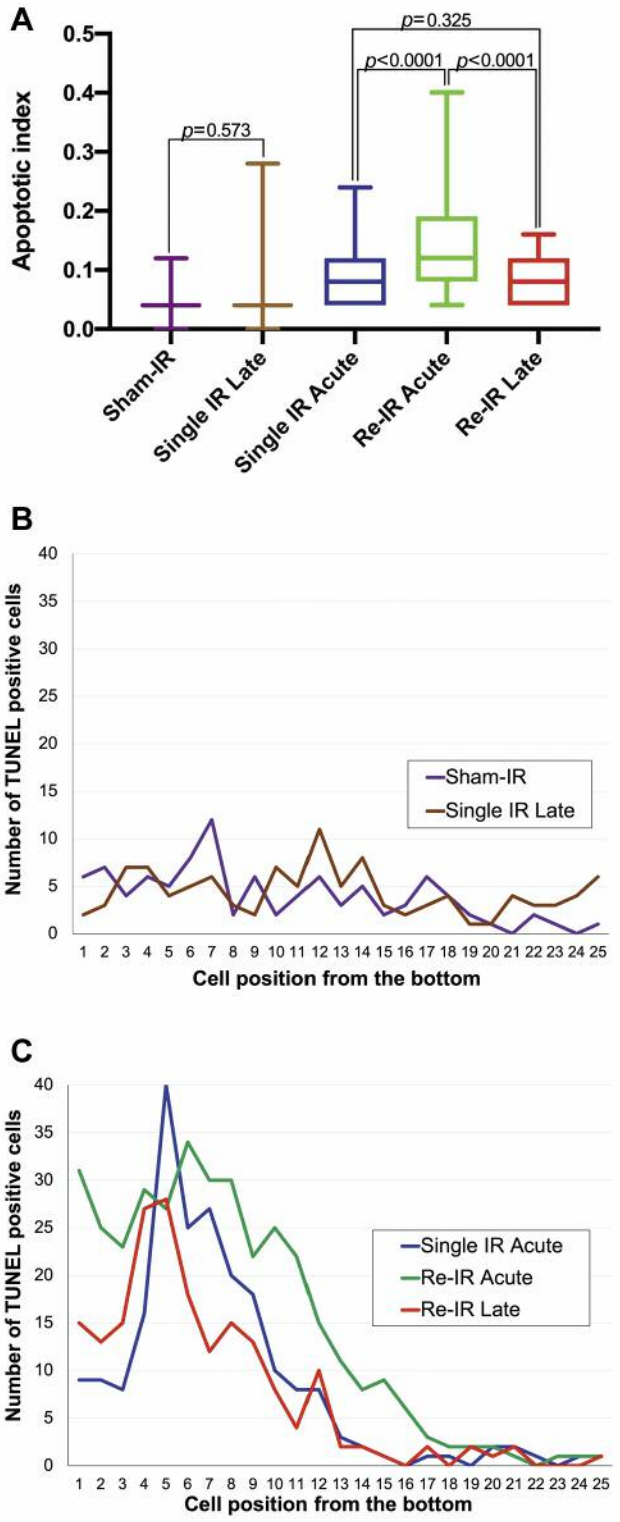


Figure 2. *Apoptotic index of each group. The apoptotic index is indicated for each group (A). The pattern of apoptotic cells after re-irradiation was assessed for each position of the columnar cells (B and C). The apoptotic index was 0.039 (0.025), 0.044 (0.036), 0.085 (0.044), 0.144 (0.076), and 0.076 (0.033) in the Sham-IR, Single IR Late, Single IR Acute, Re-IR Acute, and Re-IR Late groups, respectively.*

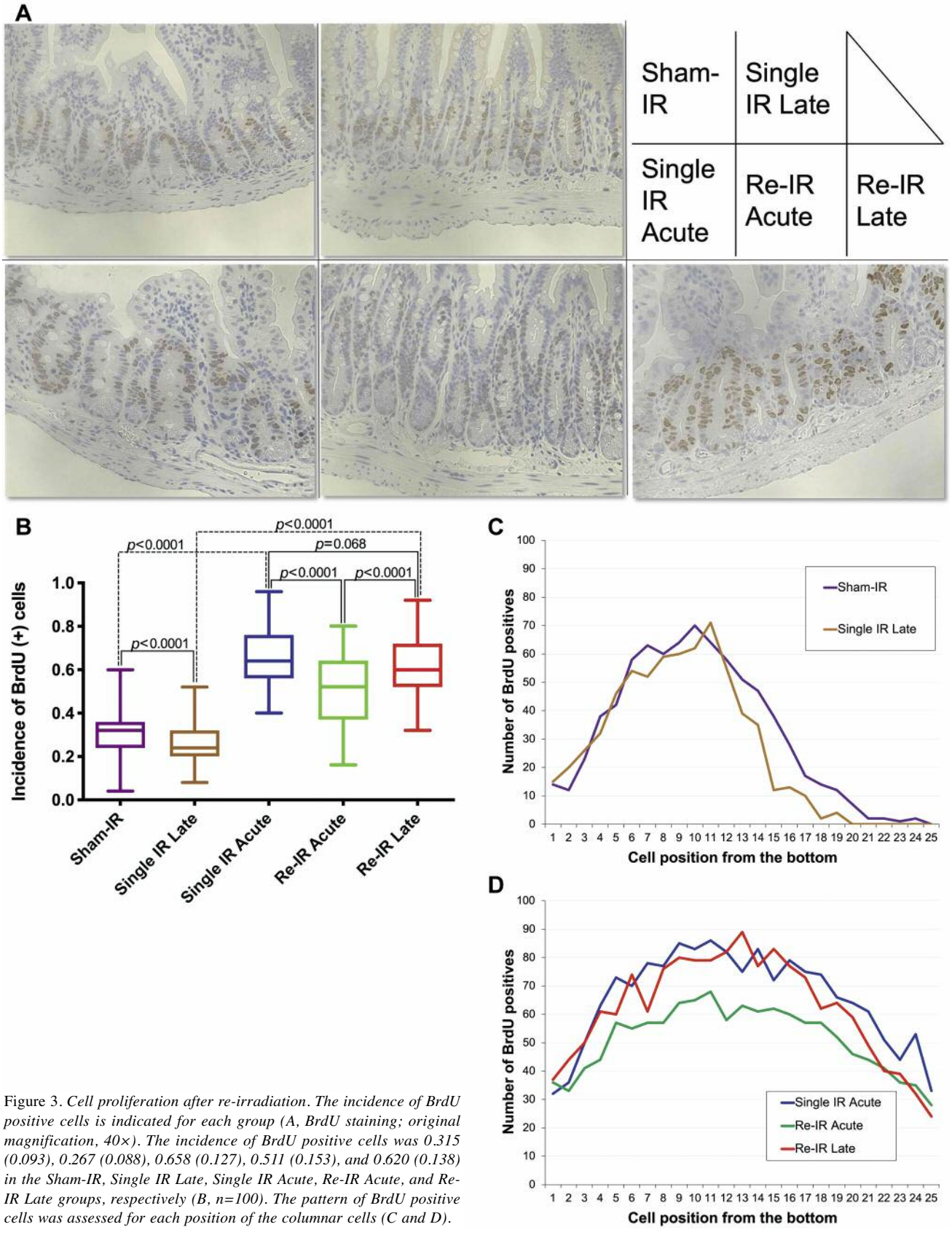


Figure 3. *Cell proliferation after re-irradiation. The incidence of BrdU positive cells is indicated for each group (A, BrdU staining; original magnification, 40×). The incidence of BrdU positive cells was 0.315 (0.093), 0.267 (0.088), 0.658 (0.127), 0.511 (0.153), and 0.620 (0.138) in the Sham-IR, Single IR Late, Single IR Acute, Re-IR Acute, and Re-IR Late groups, respectively (B, n=100). The pattern of BrdU positive cells was assessed for each position of the columnar cells (C and D).*

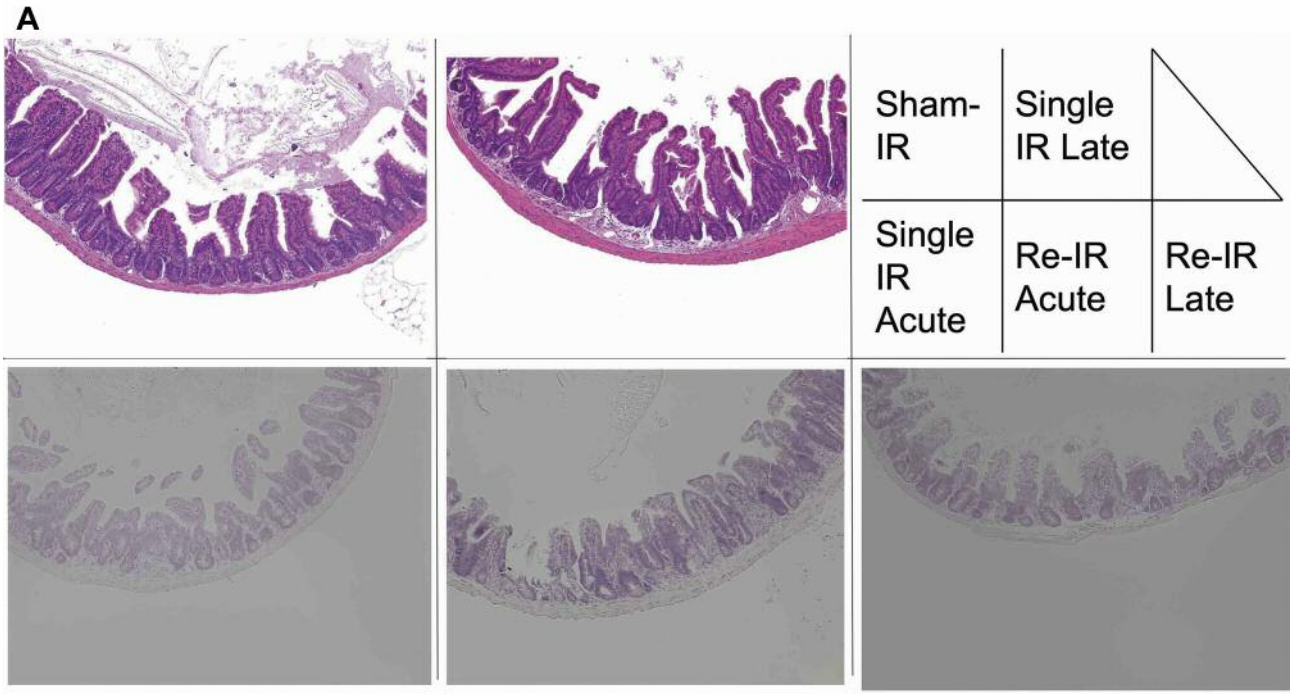


Figure 4. *Intestinal crypt stem cell assay after re-irradiation. Intestinal segments harvested from mice 3.5 days after total body irradiation (A, Hematoxylin and eosin staining; original magnification, 10×). The number of viable crypts per cross-section was quantified (B). The number of surviving crypts was 124.1 (10.2), 131.1 (26.0), 86.4 (33.2), 80.6 (33.4), and 81.6 (31.9) in the Sham-IR, Single IR Late, Single IR Acute, Re-IR Acute, and Re-IR Late groups, respectively.*

irradiation administered 2-12 months after the first irradiation was lower in re-irradiated mice in comparison with mice that received single irradiation alone, which is consistent with the findings of the present study (18). In addition, we aimed to compare the late radiation response caused by the combination of the first irradiation with the re-irradiation in the normal intestine to the acute radiation response. We believe that the 7-day and 12-week time intervals were reasonable to assess the acute and late radiation effects on normal intestine (18-20, 25), and that this experimental model can be used in the future to study potential radioprotective drugs, as well as the effect of using various clinically relevant treatment schedules.

Secondly, we used a relatively low dose of 12 Gy in a single fraction for the crypt survival assay and delivered radiation at a dose rate of 35.2-39.6 cGy/min, that is lower than that of linear accelerators used to treat patients. Since normal tissues are better able to repair low dose rate radiation damage, it is conceivable that the dose rate used in our experiments may have induced less damage than would have been observed with much higher dose rate irradiation (26). Nevertheless, submucosal fibrosis and a reduction in the number of surviving crypt cells (Figures 4 and 5) was observed as expected for the dose of radiation delivered. Moreover, fractionated irradiation which is more likely used in a clinical setting might be used in further studies since time factor can affect the responses to irradiation in both tumor and normal tissues (27). Lastly, as only acute responses were assessed in this study following re-irradiation, future experiments should include assessment of late effects of re-irradiation as well.

Results from this study have clinical relevance and suggest that re-irradiation of the intestine after an extended interval may

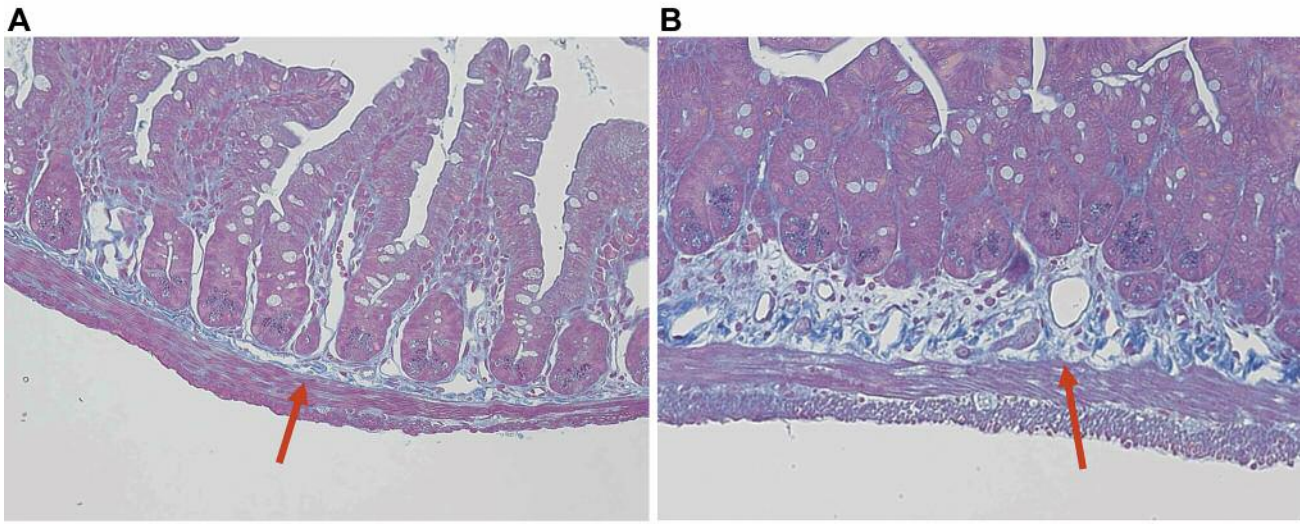


Figure 5. *Submucosal fibrosis 12 weeks after radiation exposure. Arrows indicate submucosal fibrosis in the mice (Hematoxylin and eosin staining; original magnification, 40×). Mice sacrificed 12 weeks after radiation exposure (B) show more submucosal fibrosis compared with sham-irradiated mice (A).*

be a reasonable treatment option, and is associated with acute toxicity that is not worse than that expected from similar irradiation of previously unirradiated intestine. Of course, factors such as field size, total dose, fractionation, and critical structures in the field should be carefully considered in the evaluation of patients for re-irradiation. Additional preclinical experiments with extended observation periods and fractionated regimens are needed to evaluate late changes after re-irradiation, which is particularly important for patients with a life expectancy of months to years (4-6). Moreover, mechanistic studies to assess which factors affect the severity of toxicity after re-irradiation will facilitate development of optimal re-irradiation treatment schedules.

In conclusion, re-irradiation 12 weeks after the first irradiation produced similar cell death and cell proliferation patterns in the normal intestine as observed after a single radiation exposure. Re-irradiation after an extended interval may result in equivalent biological effects on normal intestine in terms of acute toxicity and be a reasonable treatment for a subset of patients.

Conflicts of Interest

The Authors declare that no conflicts of interest exist regarding this study.

Authors' Contributions

HD conceived the experiment, analyzed the data and drafted the manuscript. HD and MT carried out the experiment. MT, KN, HM and YN provided critical revision of the manuscript. HM and YN supervised the project. All read and approved the final manuscript.

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