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# Effects of Mesenchymal Stem Cells Treatment on Radiation-Induced Proctitis in Rats

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**Purpose:** There are no effective treatment methods with which to control complications of radiation proctitis with fistula or recurrent bleeding following radiation treatment for prostate, cervical, or rectal cancer. Mesenchymal stem cells (MSCs) can induce immune modification, resulting in tissue repair and regeneration. Therefore, we used a rat model of radiation-induced proctitis and observed the effects of using human placenta-derived (PD) and adipose tissue-derived (AD) MSCs.
**Materials and Methods:** Female Sprague Dawley rats were irradiated at the pelvic area with 25 Gy. We injected $1\times10^6$ cells of human PD-MSCs, human AD-MSCs, human foreskin fibroblasts, and control media into the rectal submucosa following irradiation. We sacrificed rats for pathologic evaluation.
**Results:** Fibrosis on the rectum was reduced in both MSC groups, compared to the control group. Mucosal Ki-67 indices of both MSC injected groups were higher than those in the control group. Although caspase-3 positive cells in the mucosa gradually increased and decreased in the control group, those in both MSC injected groups increased rapidly and decreased thereafter.
**Conclusion:** We demonstrated the effects of regional MSC injection treatment for radiation-induced proctitis in rats. MSC injection reduced fibrosis and increased proliferation in rat mucosa. Human AD-MSCs and PD-MSCs had similar effectiveness.

**Key Words:** Proctitis, radiotherapy, mesenchymal stem cells, rat model

## INTRODUCTION

The incidence of radiation proctitis, which is a serious complication of prostate or cervical cancer following radiation therapy, varies from 2%–39% depending on the radiotherapy dose and technique.[1] Serious complications, such as rectal stenosis due to mucosal fibrosis and edema, neovascular bleeding, or rectovaginal fistula, decrease quality of life. Radiation proctitis, a locoregional disorder, has been treated with aminosalicylic acid derivatives, corticosteroids, sucralfate, bipolar electrocoagulation, short chain fatty acids, hyperbaric oxygen, and argon laser, albeit with no apparent success. Argon plasma coagulation appears to exert some effects on hemorrhagic radiation proctitis by breaking down neovascularization; however, this is limited to cases of stenosis or fistula and poses some side effects. Therefore, a standard treatment of choice has not yet been established.[2]

In animal models, radiation-induced rectal mucosal damage has been effectively managed with mesenchymal stem cells (MSCs).[3,4] Additionally, radiation overexposure in prostate cancer treatment in humans has also been managed with MSCs.[5] The pathophysiology of chronic radiation proctitis is mucosal inflammation caused by ionizing radiation and fibrotic progression during convalescence. Thus, MSC therapy may be a promising treatment option.[6]

We used a rat model of chronic radiation proctitis and observed pathologic changes to evaluate the prevention effects and therapeutic mechanisms of human placenta-derived MSCs (PD-MSCs) and adipose tissue-derived (AD-MSCs). We also wanted to establish a foundation for the management of radiation-induced proctitis using treatments with MSCs.

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•The authors have no potential conflicts of interest to disclose.

## MATERIALS AND METHODS

### Animal

We used female Sprague Dawley rats (weight 200 g to 250 g, age 8 to 9 weeks) following the Institutional Animal Care and Use Committee guidelines as approved by the Institutional Ethics Committee (Rule No. 11-08-079-006). Rats were housed in cages supplied with around-the-clock water and food and maintained on a 12- to 12-hour light-dark cycle. We grouped the rats into the following four groups according to the injected materials: a placebo control group, another control human foreskin fibroblast (HFF, WI-38 cell line) group, a PD-MSC group, and an AD-MSC group. Fifteen rats were included in each group, and three rats served as an unirradiated control.

### Preparation of MSCs

Human PD-MSCs and AD-MSCs were provided by CHA Biotech, Co. Ltd. (Pangyo, Seongnam, Korea). Preparations of the human PD-MSCs and AD-MSCs were conducted in a Good Manufacturing Practice (GMP) facility, and the isolation and expansion of human PD-MSCs and AD-MSCs were performed according to Good Clinical Practice guidelines of the Master Cell Bank. Preparation of the cells has been described in a previous study.[7,8]

Placenta tissue was obtained with informed consent from healthy mother donors at CHA Bundang Medical Center (Seongnam, Korea). The placental membranes were separated from the placental body and washed in Dulbecco's phosphate-buffered saline (DPBS; Gibco, Gaithersburg, MD, USA) to remove blood. Amniotic connective tissue of the placental membrane was harvested using two slide glasses and incubated at 37°C with shaking (175 rpm) for 15 min with Hank's balanced salt solution (Gibco) containing 1 mg/mL of type I collagenase (Sigma-Aldrich, St. Louis, MO, USA), 1.2 U/mL of Dispase (Gibco), 2 mg/mL of trypsin (Sigma-Aldrich), 65 μg/mL of DNase I (Roche, Mannheim, Germany), and 1 X penicillin-streptomycin (Gibco). The viability of the isolated cells was determined by trypan blue exclusion. PD-MSCs were cultured in α-modified minimal essential medium (Hyclone, Buckinghamshire, UK) supplemented with 10% FBS (Gibco), 25 ng/mL of FGF4 (Peprotech, Rocky Hill, NJ, USA), 1 μg/mL of heparin (Sigma), and 0.5% gentamycin (Gibco) at 37°C in a humidified atmosphere containing 5% $CO_2$. FACS analysis was conducted to identify the phenotypes of the cells, and PD-MSCs at passage 6 were used in the present study.

Abdominal subcutaneous adipose tissue was harvested via liposuction performed with informed consent by a plastic surgeon under local anesthesia in the operating room. About 150 mL of adipose tissue was obtained by suction using 50-mL syringes. The liposuction tissue was transported to the GMP facility of CHA Biotech Co., Ltd. The adipose tissues were washed twice with DPBS with $Ca^{2+}$ and $Mg^{2+}$, and were isolated by enzymatic dissociation followed by centrifugation at room temperature. The cells were plated on flasks and incubated at 37°C in a humidified incubator under a 5% $CO_2$ atmosphere. The medium was changed every 2–3 days until the cells achieved 80%–90% confluence. AD-MSCs at passages 1, 3, 6, and 12 were used for cell characterization. FACS analysis was used to identify the phenotypes of the cells. AD-MSCs at passage 3 were used in the present study.

### Radiation dosage for experiment

In line with the results of previous research,[6,9] rats were irradiated with 6-MV photons from a medical linear accelerator (Clinac iX, Varian Medical Systems) with a source-to-axis distance of 100 cm. Radiation doses of 20, 23, and 25 Gy were delivered at 6 Gy per min. Rats were anesthetized with an intraperitoneal injection of 50 mg/kg of ketamine. Two rats at a time were restrained and placed in transparent plastic boxes in the supine position. Lead shielding was used to cover the rats, except for the lower pelvis containing 3 cm of the length of the rectum. After evaluating the effect of irradiation on rectal mucosal atrophy and fibrosis at 2 and 4 weeks, we chose 25 Gy as an appropriate dose (Fig. 1).

### MSC injection

We injected $1\times10^6$ human PD-MSCs or AD-MSCs with a 31-gauge needle into the submucosal layer of the rectum irradiat-

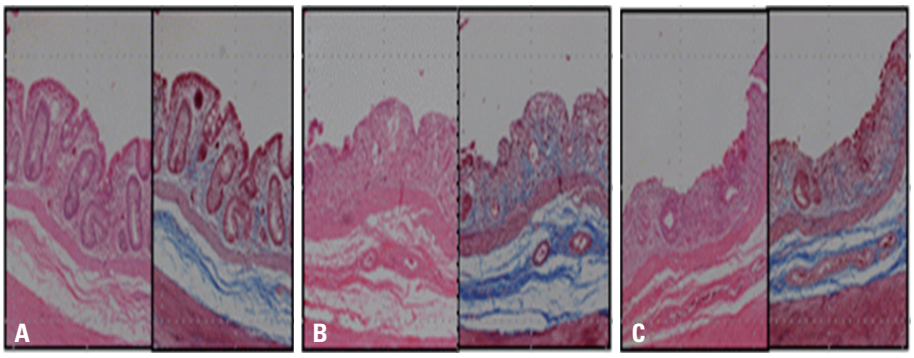


**Fig. 1.** Histologic changes in the rat rectum following 2 weeks of irradiation. Mucosal atrophy and submucosal fibrosis are noted. (A) 20 Gy irradiated group. (B) 23 Gy irradiated group. (C) 25 Gy irradiated groups (left; H&E stain, right; Masson trichrome stain).


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ed with 25 Gy at 24 hours (Fig. 2). We checked the rats' body weights for evidence of anal bleeding and rectal obstruction every week.

### Histologic evaluation of radiation-induced mucosal damage

We randomly selected rats for histologic evaluation from each group on days 1, 3, and 4 and weeks 2 and 4 following irradiation. We checked the following items during histologic evaluation under the guidance of a pathologist:

*Pathologic grading with hematoxylin and eosin (H&E) stain*

Upon staining with H&E, pathologic changes in tissues were graded as 0, normal or minor damage; 1, slight damage with mild inflammation or slight crypt changes; 2, mild damage with more significant inflammation or crypt changes; 3, moderate damage with a prominent loss of the epithelium; and 4, severe damage with signs of ulcer or necrosis.

*Fibrosis state with Masson trichrome stain*

We stained the rectal tissue with Masson trichrome stain and evaluated differences in fibrosis within each group by quantification of the fraction of fibrosis area/total tissue using Image-J software (https://imagej.nih.gov/ij/index.html).

*Identification of donor cells with immunofluorescence*

Human stem cells were taken from males and had XY chromosomes. We checked Y chromosomes with the fluorescence in situ hybridization (FISH) method.

*Immunohistochemical stain for proliferating cells and apoptosis*

We stained for Ki-67 to identify proliferating cells in the rectal mucosa. We calculated the Ki-67 index by means of the ratio between stained cells and non-stained cells in 100 rectal mucosal glands. We administered caspase-3 stain to evaluate apoptosis grade. We calculated stained cell means in 100 rectal mucosal glands.

### Living image software (Xenogen®) assay for donor cells

Stem cells were prepared by tagging them with 3-(chloromethyl) benzamide (CellTracker™ CM-DiI: C700) and the red fluorescent carbocyanine tracer DiD [$DiIC_{18}$(5)-DS: D12730] before injection. We checked the presence of injected stem cells with Xenogen (Caliper Life Science, Hopkinton, MA, USA) from the resected rectum of rats at 2 and 4 weeks.

### Data analysis

Measured value data are summarized as a mean±standard deviation. We evaluated characteristic differences with Student's t-test and evaluated categorized data with $\chi^2$ analysis. *p* values below 0.05 were considered statistically significant.

## RESULTS

### Characteristics of MSCs

In this study, we used MSCs with XY chromosomes after six passages. Cell surface antibodies showed CD44 (+), CD 73 (+), CD 105 (+), CD 34 (-), CD 31 (-), and CD 45 (-). Cell sizes of AD and PD MSCs were 15.6 μm and 14.5 μm on average, respectively (Fig. 3).

### Clinical course following irradiation

We did not observe weight gain in the rats for the first 3 days following irradiation. We checked their weight for four weeks. The AD-MSC group, PD-MSC group, HHF group, and radiation control group had weight gains of 51.5±17.8 g, 68.5±11.1 g, 66.2±14.9 g, and 68.2±17.0 g, respectively. All these weight gains were

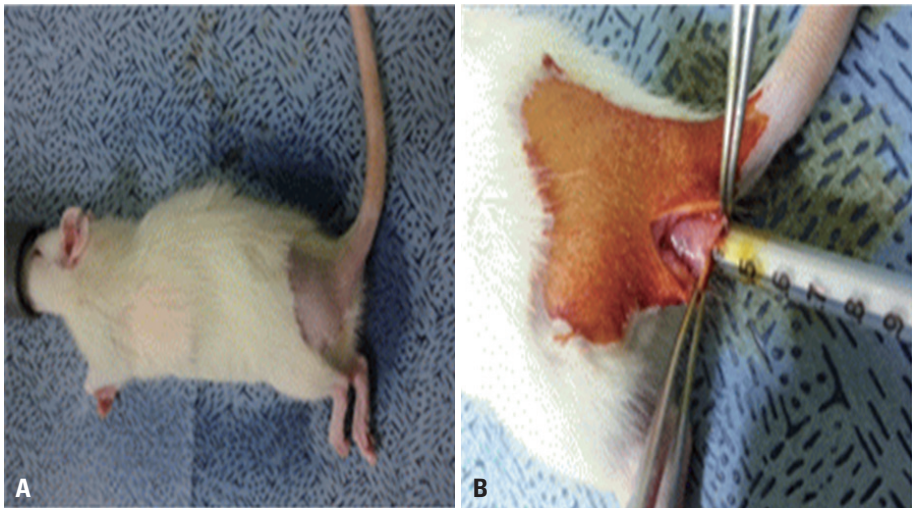


**Fig. 2.** Injection of human placental-derived or adipose tissue-derived mesenchymal stem cells. Following general anesthesia with inhalation, (A) the external side of the rectum was exposed after partial incision of the skin and muscle layer. (B) Stem cells were injected with a 1-cc syringe and a 31-gauge needle.

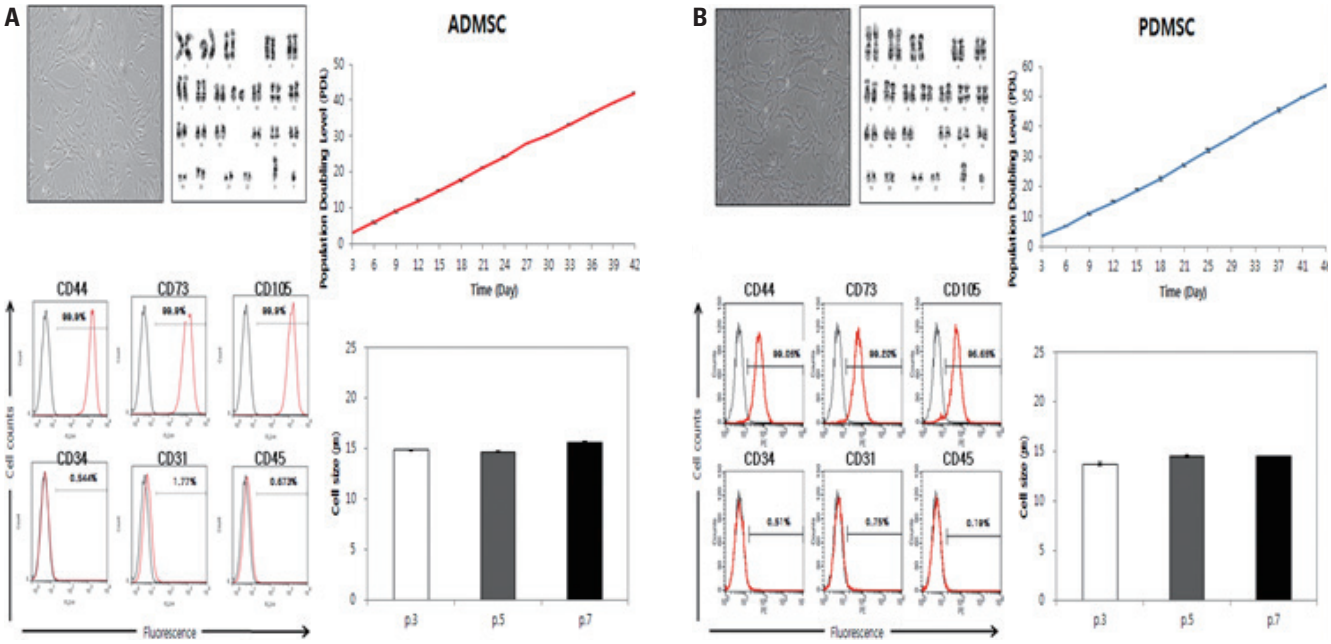


**Fig. 3.** Characteristics of AD-MSCs and PD-MSCs. (A) AD-MSC. (B) PD-MSC. They all had DNA XY genes and showed CD 44 (+), CD 73 (+), CD 105 (+), CD 34 (-), CD 31 (-), and CD 45 (-). AD-MSC, adipose tissue-derived mesenchymal stem cell; PD-MSC, placenta-derived mesenchymal stem cell.

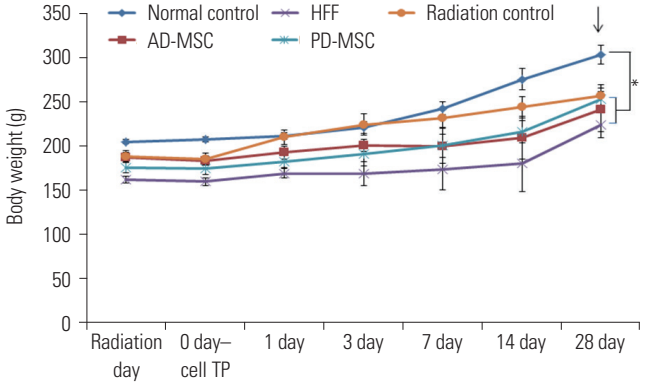


**Fig. 4.** Weight changes according to time. Compared to control group weights, the weights of the AD-MSC group, PD-MSC group, HHF group, and radiation control group were significantly lower. *$p$<0.001. AD-MSC, adipose tissue-derived mesenchymal stem cell; PD-MSC, placenta-derived mesenchymal stem cell; HFF, human foreskin fibroblast.

statistically less than those (98.7g) of the control group ($p$<0.001) but did not differ between groups ($p$=0.114) (Fig. 4). All rats were alive for 4 weeks, and diarrhea or anal bleeding did not differ statistically between groups.

### Histologic change following irradiation

Infiltration of inflammatory cells into the mucosa, destruction of glands, and mucosal layer atrophy were found on the first, third, and seventh days following irradiation. The appearance of atypical glands and regeneration of epithelium were noticed on week 2. These changes were more prominent on week 4.

### Identification of stem cells in the tissue

Cell injection sites were found at the submucosal layer of the rectum with H&E stain and Masson trichrome stain (Fig. 5). We noted high signals from the rectal tissue in the PD-MSC and AD-MSC groups with Living Image Software (Xenogen®). These signals were present on week 4, but decreased in density relative to those on week 2 (Fig. 6). Human Y chromosomes were found in the rats' rectal tissue on imaging with FISH for a Y chromosome on week 2 following irradiation (Fig. 7).

### Fibrosis state

Area fractions of fibrosis were 70.2%±3.9% on week 1, 61.5%±1.5% on week 2, and 55.4%±1.5% on week 4 in the control group. They were 48.5%±1.3% on week 1, 43.8±2.4% on week 2, and 40.6%±1.3% on week 4 in AD-MSC group, and they were 35.6%±2.5% on week 1, 40.7%±1.9% on week 2, and 31.6%±5.3% on week 4 in PD-MSC group. These scores were significantly lower in stem cell injection groups than in the control and HFF groups on week 4 ($p$<0.01) (Fig. 8).

### Proliferation state

Ki-67 proliferation indices at the rectal mucosa on weeks 2 and 4 were 52%±3.2% and 39%±12.0% in the control group, 59%±5.8% and 55%±5.4% in the AD-MSC group, and 60%±3.9% and 65%±2.6% in the PD-MSC group, respectively. Ki-67 proliferation indices were higher in the stem cell injected groups than in the control group on weeks 2 and 4 ($p$<0.01) (Fig. 9).

### Apoptosis state

We calculated caspase-3-positive cells on each gland of the rectal mucosa following stem cell injection. In the control group, these counts gradually increased until day 14 and then decreased thereafter. However, caspase-3-positive cells in the stem cell-in-


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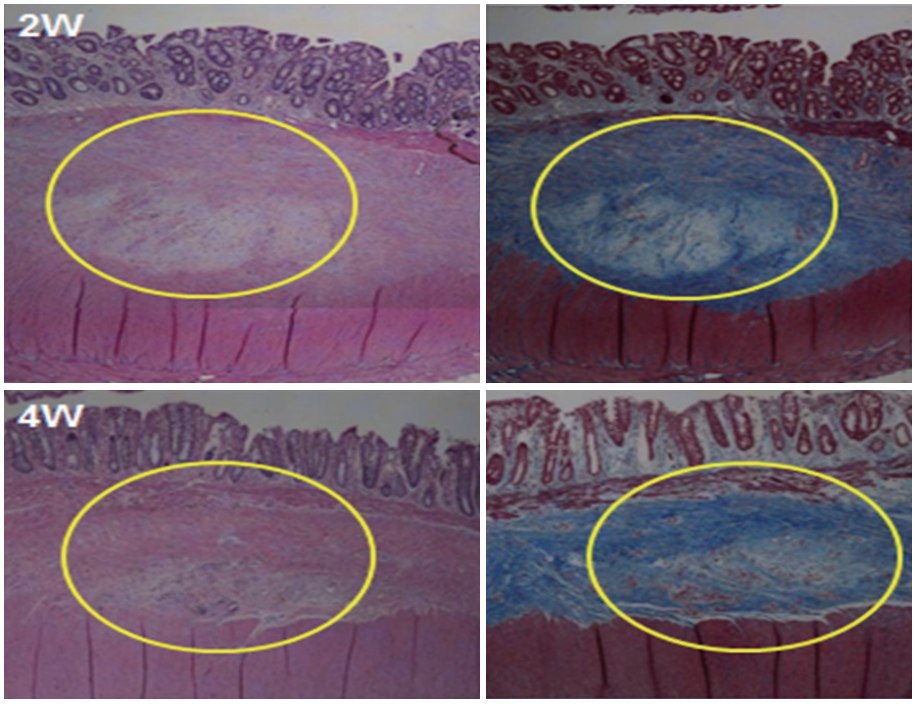


**Fig. 5.** Histologic images of the rectum on the second- and fourth weeks following injection of AD-MSCs (H&E stain and Masson trichrome stain, ×100). Cell injection sites (yellow circle) are found at the submucosal layer. AD-MSC, adipose tissue-derived mesenchymal stem cell.

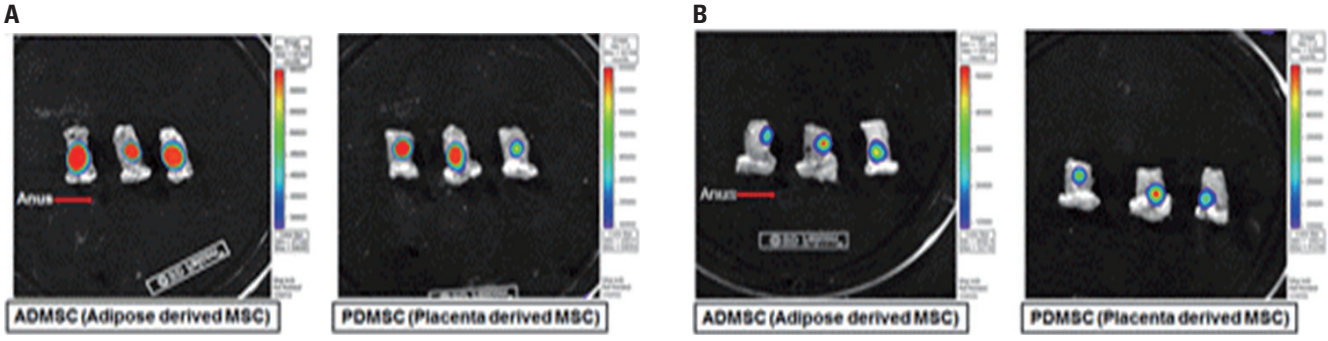


**Fig. 6.** Rectal tissue image obtained with Living Image Software (Xenogen®). Signals from the injected cells (AD-MSC and PD-MSC) were found on week 2 (A) and week 4 (B). AD-MSC, adipose tissue-derived mesenchymal stem cell; PD-MSC, placenta-derived mesenchymal stem cell.

jected groups increased more rapidly on day 3 and decreased thereafter (Fig. 10).

## DISCUSSION

Histologic changes in radiation-induced proctitis include mucosal damage and atrophy caused by inflammatory cells infiltration in the acute stage, as well as tissue ischemia and fibrosis because of endothelial cell swelling and endarteritis in the subacute and chronic stage.[1] Therefore, chronic complications, such as mucosal hemorrhage, ulcer, stenosis and fistula, may occur following radiation of the cervix, prostate, and rectum.

MSCs can trigger immune modulation, neovascularization, and anti-fibrosis. Therefore, stem cell therapy may offer a novel strategy for the treatment of radiation-induced complications. There is a report of radiation proctitis treated with stem cell therapy.[5] In the report, radiation accidents at a public hospital in France affected 425 patients with prostate cancer. Clinical consequences of overdosed irradiation were severe. Three patients received intravenous infusions of MSCs ($5\times10^6$/kg) from family donors for the treatment of hemorrhagic fistulizing colitis. They showed positive clinical responses. To evaluate the strategy of stem cell therapy in radiation proctitis, we used a radiation-induced animal model and treated it with MSCs in this study and obtained positive results.

MSCs influence antigen presenting cells to change the cytokine profiles of T-cells, NK cells, and dendritic cells from pro-inflammatory phenotypes to tolerant phenotypes. MSCs have immunosuppressive effects on dendritic cells that decrease secretion of tumor necrosis factor-α, interferon-γ, co-stimulatory molecules (CD 80 and CD 86), as well as on T-cells to inhibit

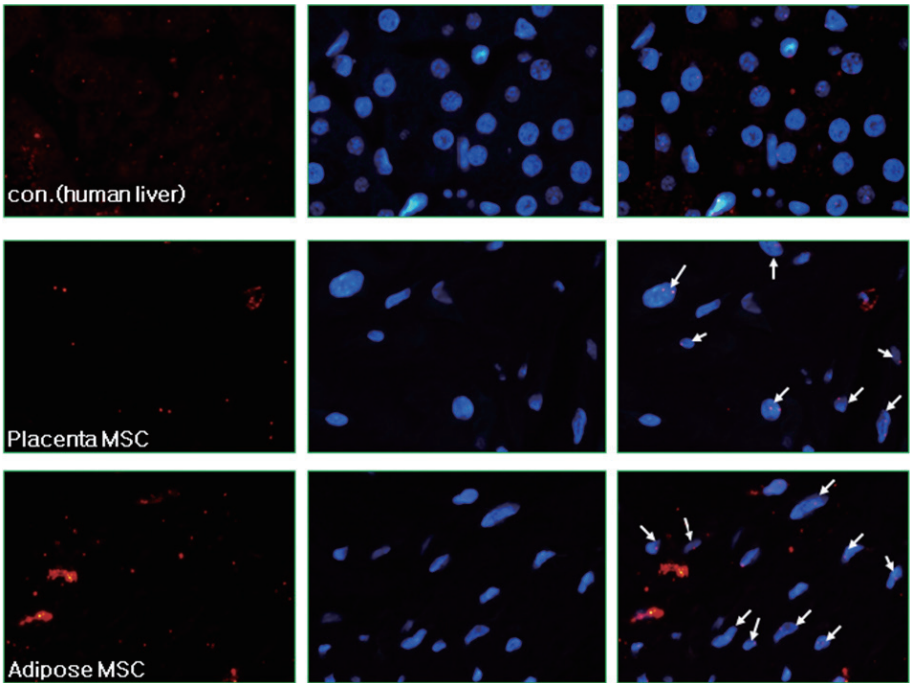


**Fig. 7.** Image of fluorescence in situ hybridization for human Y chromosome in the rectal tissue of rats on week 2 following irradiation. Y chromosomes (white arrows) were found. MSC, mesenchymal stem cell.

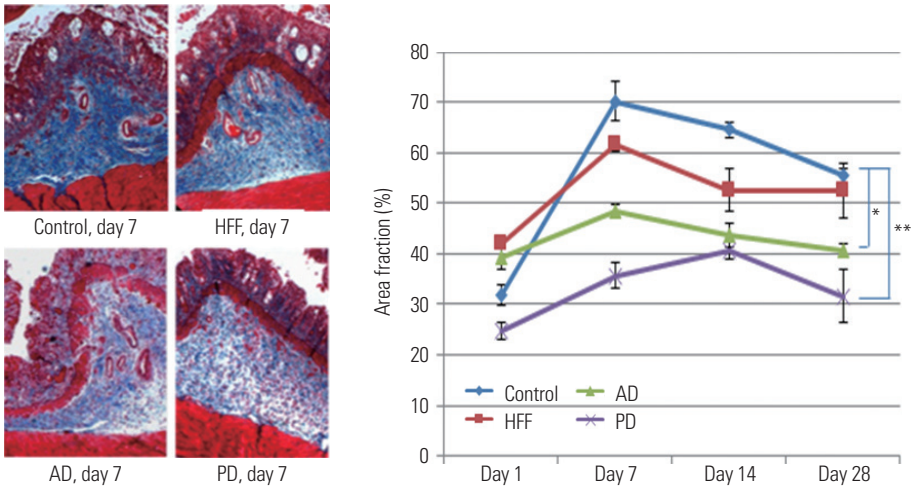


**Fig. 8.** Images of fibrosis with Masson trichrome stain. Area fractions of fibrosis in mesenchymal stem cell injection groups were significantly lower than those in the control or HFF groups on week 4. *,**$p<0.01$. AD, adipose tissue-derived; PD, placenta-derived; HFF, human foreskin fibroblast.

differentiation and proliferation, and finally on T-regulatory cell lines to activate secretion of transforming growth factor-β, interleukin-10, and prostaglandin E2. MSCs are pluripotent cells that can differentiate into neural cells, adiposities, bone, and epithelial cells, and they can migrate to damaged tissues, such those caused by irradiation. They can even help to regenerate tissues.[10,11]

MSC treatment has been performed in other organs. Treatment with placental chorionic plate-derived MSCs in a rat model of hepatic fibrosis elicited antifibrotic effects by inhibiting collagen I synthesis and by triggering MMP-2 and MMP-9 activation.[12] In addition, transplantation of MSCs has been found to be effective in the ischemic myocardium.[13] In addition to placental and adipose tissue, bone marrow-derived MSCs have been used in treatment of radiation-induced intestinal damage in mice, inducing regeneration of intestinal mucosa through intestinal stem cell differentiation, reduction of apoptosis and inflammation, and increased secretion of growth factors and cytokines.[14-19]

There are some differences between prior studies and this study of stem cell treatments for radiation-induced intestinal injury. First, previous studies reported intravenous or intraperitoneal systemic injection of stem cells following whole body irradiation.[14-19] However, most radiation treatment for cervical,


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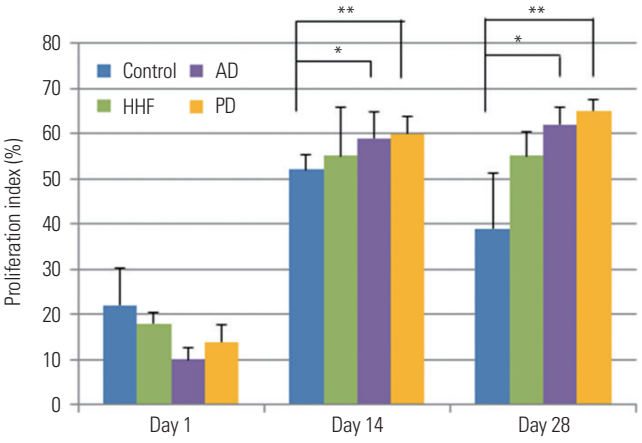


**Fig. 9.** Ki-67 proliferation index values of the rectal mucosa on weeks 2 and 4 following irradiation. *,**$p$<0.01. AD, adipose tissue-derived; PD, placenta-derived; HFF, human foreskin fibroblast.

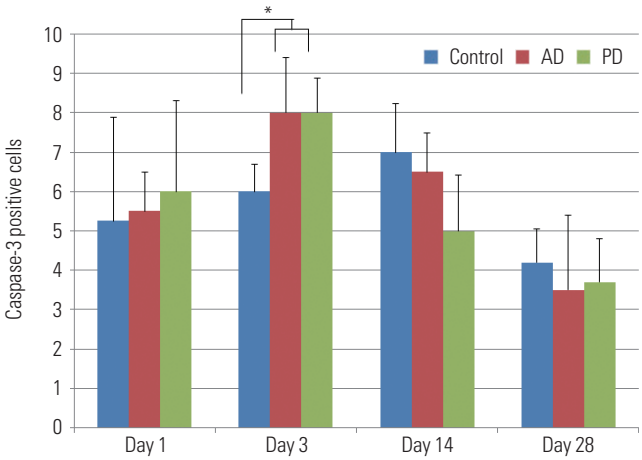


**Fig. 10.** Caspase-3 positive cells in each rectal mucosal gland following stem cells injection in each group. *$p$<0.05. AD, adipose tissue-derived; PD, placenta-derived.

prostate, or rectal cancers focus on the lower pelvis, as well as injured sites in the rectum. Therefore, the present study used a regional irradiation model. According to a previous study, sequential changes occur following irradiation. Acute inflammation develops just after irradiation and is most prominent on days 10 to 14. Thereafter, regeneration starts and radiation injury regresses in 4 to 8 weeks, or it changes to chronic proctitis.[20] A rat model for radiation-induced proctitis reported 17.5 Gy as being adequate as a radiation dosage.[6] Usually, irradiation below 15 Gy induces minimal histologic changes, whereas when it is above 20 Gy, it induces serious mucosal changes. Regeneration is naturally observed about 4 weeks following irradiation and cannot be differentiated from stem cell effects thereafter. In this study, we used 25 Gy, which is higher than that in previous rat models, and histologic injury was severe. However, mortality or serious clinical symptoms were not observed because of regional reaction.

Second, in this study we used regional injection of MSCs instead of systemic injection. Because large portions of systemically injected stem cells are harvested in the lung and liver and only small portion of them go together to injured tissues.[21] In addition, in contrast to systemic Crohn's disease, radiation proctitis is a local disease. Thus, systemic injection is ineffective.

Third, previous studies used bone marrow-derived MSCs[3,5] and human amnion-derived MSCs.[4] The multilineage differentiation potentials of PD-MSCs are similar with those of bone marrow-derived MSCs. Human PD-MSCs confirm more closely to ethical standards and can be obtained in sufficient numbers without invasive processes, such as bone marrow aspiration.[22] In this study, human AD-MSCs exhibited similar effectiveness to PD-MSCs.

Fourth, in contrast to short-term evaluation in previous studies, this study evaluated acute injury of radiation proctitis and regeneration processes over 4 weeks of follow up. We documented transplanted stem cell tissue signals for 4 weeks in a gradual tapering pattern using an image assay method. On evaluation, MSC transplantation showed a significant effect on the regeneration process of antifibrosis, apoptosis, and proliferation indices at the second and fourth weeks.

The main pathophysiology of chronic radiation proctitis is mucosal inflammation and fibrosis of the regeneration process caused by ionizing radiation. In this study, submucosal fibrosis was noticed after irradiation and was ameliorated in the MSCs injection group. In a rat liver cirrhosis model, PD-MSCs inhibited fibrosis by decreasing synthesis of collagen I and by increasing synthesis of MMP-2 and MMP-9.[12,22] Transplantation of MSCs was effective in many fibrosis related disorders[10] and injection of MSCs to radiation proctitis is expected to be a possible treatment modality.

When stem cells are injected into the area of radiation injury, epithelial proliferation increases with injection.[15,19] We used a Ki-67 stain in this study to identify proliferative cells, which were higher in the MSCs injection groups than the control or PBS group, especially during the second and fourth weeks of the regeneration process. This suggests that MSC injections may help in the regeneration of innate epithelium.

Ionizing radiation has anti-oncotic effects by inducing apoptotic cell death. The exact mechanism is not well known, but p21, caspases, BAX, and BCL-2 genes activation is associated. Caspase-1 and -10 are associated with chemical and physical agent-induced apoptosis, whereas caspace-3 is associated with radiation-induced apoptosis. Caspase-3 activity increases in early phase of radiation.[23,24] In this study, caspase-3-positive cells in all rectal mucosal glands gradually increased for 2 weeks following radiation injury and then decreased thereafter. Interestingly, those in the MSC-injected groups increased earlier on day 3 and then decreased. Further studies on the quantification and mechanisms of radiation-induced apoptosis are needed.

In conclusion, we employed a rat model of radiation-induced proctitis to evaluate treatment with regional injection of MSCs for radiation proctitis. Our results indicate that injection of MSCs reduces fibrosis and increases proliferation in the mucosal layer

and support further exploration of MSCs as a potential treatment modality for treating radiation-induced proctitis.

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## AUTHOR CONTRIBUTIONS

Conceptualization: Sung Pyo Hong and Won Hee Kim. Data curation: Won Hee Kim. Formal analysis: Jun Hwan Yoo and Chang Il Kwon. Funding acquisition: Sung Pyo Hong. Investigation: Won Hee Kim and In Kyung Yoo. Methodology: Won Hee Kim and Jun Hwan Yoo. Project administration: Jun Hwan Yoo and Chang Il Kwon. Resources: Won Hee Kim and In Kyung Yoo. Software: Won Hee Kim and In Kyung Yoo. Supervision: Sung Pyo Hong. Validation: Sung Pyo Hong, Won Hee Kim, In Kyung Yoo, and Chang Il Kwon. Visualization: Chang Il Kwon. Writing—original draft: Won Hee Kim and Jun Hwan Yoo. Writing—review & editing: Sung Pyo Hong and Jun Hwan Yoo. Approval of final manuscript: all authors.

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