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A Rat Model of Radiation Vasculitis for Mesenchymal Stem Cell-based Therapy

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Dear Editor,

It is my great pleasure to submit my paper entitled “a rat model of radiation vasculitis for the study of mesenchymal stem cell-based therapy” to your journal. Our purpose is to improve local recruitment of mesenchymal stem cells by modifying conventional models. The new model is based on the theory that local irradiation promotes the recruitment of mesenchymal stem cells to the field of irradiation. The new model will find its application in the study of mesenchymal stem cell-based therapy for radiation vasculitis. We think that the content of the paper is suitable for publication in your journal. Please feel free to contact us if you have any queries. We are looking forward to your comments on the paper.

Yours sincerely,

Wei Wei

TITLE:

A Rat Model of Radiation Vasculitis for Mesenchymal Stem Cell-based Therapy

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KEYWORDS:

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SUMMARY:

A rat model, namely radiation plus transplantation, was introduced to promote the homing of intravenously injected mesenchymal stem cells to an irradiated vascular segment. The model found its application in the assessment of mesenchymal stem cell therapy for radiation vasculitis.

ABSTRACT:

Radiation vasculitis is one of the most common detrimental effects of radiotherapy for malignant tumors. This is developed at the vasculature of adjacent organs. Animal experiments have shown that the transplantation of mesenchymal stem cells (MSCs) restores vascular function after irradiation. However, the population of MSCs migrating to irradiated vessels is too low in the conventional models, which makes an assessment of the therapeutic effect difficult. This is presumably because circulating MSCs are dispersed in adjacent tissues that are being irradiated simultaneously. Based on this assumption, a specific rat model, namely RT (radiation) plus TX (transplantation), was established to promote MSC homing by sequestering irradiated vessels. In this model, a 1.5 cm-long segment of rat abdominal aorta was irradiated by a 160 kV X-ray at a single dose of 35 Gy before being procured and grafted to the healthy counterpart. F344 inbred rats served as both donors and recipients to exclude the possibility of immune rejection. A beam-limiting device was used to confine the X-ray beam to a 3 x 3 cm square-shaped field covering

the central abdominal region. The abdominal viscera, especially the small bowel and colon, were protected from irradiation by being pushed off the central abdominal cavity. Typical radiation-induced vasculopathy was present on the 90th day after irradiation. The recruitment of intravenously injected MSCs to the irradiated aorta was significantly improved by using the RT-plus-TX model as compared to the model with irradiation only. Generally, the RT-plus-TX model promotes MSC recruitment to the irradiated aorta by separating the irradiated vascular segment from the adjacent tissue. Thus, the model is preferred in the study of MSC-based therapy for radiation vasculitis when the evaluation of MSC homing is demanding.

INTRODUCTION:

Radiotherapy is used to treat a variety of cancers, but the therapeutic index of radiotherapy is still limited by normal tissue injury in organs at risk. Vascular injury is the major cause of late radiation morbidity. Patients with head and neck tumors have a higher risk of developing dementia and cognitive dysfunction after radiotherapy[1,2]. Despite direct ionizing radiation injury to neurons and glial cells, brain blood circulation disorder resulting from vascular injury is an essential contributory factor[3,4]. The small bowel is very vulnerable to irradiation. Chronic radiation enteritis is initiated as early as 2 months after patients received abdominal/pelvic irradiation, progressing throughout the rest of their life[5]. This is characterized by progressive obliterative arteritis with submucosal fibrosis[5]. Typically, the irradiated vessels develop slowly toward vascular fibrosis with luminal stenosis, excessive extracellular matrix deposition in the media and adventitia, intimal hyperplasia, and thrombus formation[4]. The process has been replicated by rat models, in which radiation-induced vasculopathy is present 3 - 6 months after irradiation[6,7]. Many potential therapeutic strategies have been investigated in these models prior to their clinical use.

Stem cell therapy holds great promise for radiation-induced vascular injury. MSCs are multipotent stromal cells that can differentiate into a variety of mature cell types. Moreover, MSCs themselves secrete a broad spectrum of trophic factors that serve to structure regenerative microenvironments[8]. MSCs were first separated from bone marrow and later found in other mesenchyme tissue. Their ease of isolation and manipulation and their potential use for tissue regeneration are specifically what has made them so attractive[9]. A number of animal studies have demonstrated that MSCs restore vascular function by both intravascular injection and seeding of vascular graft[6,8,10-12]. Nevertheless, it has been rarely reported how many circulating MSCs exactly reach irradiated vessels after intravascular injection, presumably because the recruited cell number is fairly low. This is a practical problem in MSC-based therapy for radiation injury and other diseases as well[8]. In a sense, the scarcity of MSCs in irradiated vessels renders the assessment of therapeutic effect somewhat difficult, given that MSC recruitment is a prerequisite for effective cell-based therapy[13]. One possible explanation for the phenomenon is that transplanted MSCs are dispersed in adjacent tissues, which are inevitably irradiated but constitute a large compartment of MSC recruitment. In that case, the effort to localize radiation exposure probably enable MSCs to aggregate in irradiated vessels. The assumption is supported by a previous investigation of the quantitative and spatial distribution of infused MSCs after local irradiation. Total body irradiation-stimulated MSCs homed at a very low level to various tissues of the whole body with additional local irradiation resulted in significant MSC engraftment in the

exposed area[14]. Inspired by the findings, this study introduces a rat model, namely RT plus TX, in which irradiated vessels are sequestered from adjacent tissue to promote MSC local recruitment.

PROTOCOL:

All animal procedures were approved by the Committee of Animal Experiment Ethics at Nanjing Medical University (Nanjing, China).

1. Animal and Cell Lines

1.1. Use female F344 rats at 12 weeks of age for the surgical procedures in this protocol, like abdominal irradiation and aorta transplantation, and as the host for MSC transplantation. Raise the rats in a specific pathogen-free grade barrier facility with a 12/12 h light/dark cycle, the temperature at 18 - 22 °C, and the relative humidity at 40% - 60%. Feed the rats with standard pelleted food and water.

1.2. Include 48 rats in the study. Divide them evenly into six groups according to the treatment protocols (**Table 1**).

1.3. Use male F344 rat MSC cell lines for the cell transplant. Culture the cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C with a humidified atmosphere of 5% CO_2 and 95% air.

NOTE: MSC transplantation from male donors to female recipients enables cell tracing by the sex determination region on the Y chromosome (Sry).

2. Abdominal Irradiation

2.1. Inject the rat with 10% chloral hydrate solution at a single dose of 0.3 mL per 100 g body weight *via* the intraperitoneal route. Wait 5 min to assess the anesthetic depth using reflex testing.

2.2. Fix the rat in a supine position with its four legs stretched outward. Prep the abdominal skin by shaving its hair and disinfecting it with 70% ethanol (EtOH).

2.3. Make a 5 cm midline incision by using scissors to open the abdominal cavity. Pull out the small bowel and the colon by using saline-soaked cotton swabs, and leave them to the right of the abdominal cavity, beyond the field of irradiation. Place the small bowel and colon on a piece of gauze that is wetted with warm saline to keep them moist.

2.4. Transfer the rat to the chamber of a biological irradiator. Make sure the chamber door is securely closed throughout the irradiation process. There is no asphyxiation as the chamber is not airtight.

2.5. Irradiate the rat in a ventrodorsal direction with a 160 kV X-ray, operating at 25 mA and filtered with 0.3 mm of copper. Set the total irradiation dosage to 35 Gy at the rate of 1.75 Gy/min.

2.6. Localize the irradiation to a square-shaped field of 3 x 3 cm, encompassing the central abdominal region by using a beam-limiting device or a lead shield. Leave the viscera, especially the small bowel and the colon, off of the irradiation field, to avoid the devastating gastrointestinal adverse effect.

2.7. Open the chamber door and take the rat out after the completion of irradiation.

2.8. Push the small bowel and colon back into the abdominal cavity. Close the abdominal incision with a 3-0 polyglactin suture for the rats of the RT-only model, which are not subjected to aortic transplantation. Keep the rats in a warming blanket until they have recovered from the anesthesia.

3. Aorta Transplantation

3.1. Sterilize all surgical instruments before use.

3.2. Keep the rat fixed on the operating table after the completion of the irradiation. Note that an additional injection of 10% chloral hydrate solution is not necessary since the rat is still under anesthesia.

3.3. Push the small bowel and the colon aside to expose abdominal aorta by using a Colibri retractor. Perform most of the following surgical procedures under a stereomicroscope.

3.4. Carefully dissect the infrarenal aorta away from adjacent tissue by using two microtweezers. Ligate lumbar arteries branching from the aorta with 9-0 nylon sutures.

3.5. Procure a 1.5 cm-long aortic graft by cutting with microscissors when its blood flow is blocked by the ligation right below the infrarenal artery and at aortic bifurcation.

3.6. Perfuse the aortic graft with 125 U/mL heparin solution by using a 2 mL disposable syringe with a 26 G hypodermic needle to wash the vessel clear of all blood components. Store the graft at 4 °C before use.

3.7. Euthanize the donor rat by cervical dislocation.

3.8. Take a healthy rat as the recipient. Follow step 2.1 to administer anesthesia, step 2.2 for skin prepping, and steps 3.3 and 3.4 to expose and separate the abdominal aorta.

3.9. Temporarily block the blood flow of the aorta by inserting two microclamps: one right below the renal branch and the other at aortic bifurcation.

3.10. Transect the abdominal aorta with microscissors at the midpoint of the renal arteries and aortic bifurcation. Rinse the cut ends with heparin solution by using a 2 mL disposable syringe with a 26 G hypodermic needle.

3.11. Anastomose the graft aorta to the recipient aorta in an end-to-end manner by running stitches with a 9-0 nylon suture. Use a microneedle holder and microtweezers to perform the anastomosis.

3.12. Remove the microclamps to restore the blood flow.

3.13. Use one of the following two hemostat methods if anastomotic bleeding occurs.

3.13.1. Simply press the anastomosis with a dry cotton swab for 30 s if the bleeding is not serious.

3.13.2. Apply tissue adhesive to stop the bleeding if it is not easy to control.

3.13.2.1. Use the microclamps again to stop the bleeding and apply a 0.5 µL aliquot of tissue adhesive along the anastomotic line with a 2 µL pipette.

3.13.2.2. Wait 10 s to form a strong and transparent layer of hemostat covering around the anastomosis. Take the microclamps away to check the patency and bleeding of the anastomosis.

3.13.2.3. Keep in mind that applying too much adhesive results in anastomotic stenosis and subsequent thrombus formation.

3.14. Close the abdominal incision by a running 3-0 polyglactin suture.

3.15. Keep the recipient in a warming blanket until it has recovered from the anesthesia.

3.16. Inject 1 mL of normal saline *via* the tail vein if blood loss is estimated to exceed 2 mL.

3.17. Feed the recipient on water and a normal diet after the operation.

3.18. Euthanize the recipient on postoperative day 90 to procure the graft aorta for biomedical analysis. Please note that the vascular anastomoses are carefully removed to avoid the effect of suture material on the evaluation of the vasculopathy.

4. MSC Infusion

4.1. Label MSCs with green fluorescence protein (GFP) by viral transfection, according to the a previous report[15]. Analyze the expression of MSC markers (positive for CD90, CD44, and CD29, and negative for CD34, CD45, and CD11b/c) to make it sure that there are no significant changes after the viral transfection (**Table 2**).

4.2. Prepare fresh MSCs in serum-free medium. Follow step 2.1 to anesthetize the rats for the MSC infusion.

4.3. Infuse MSCs *via* tail vein to each rat at a dose of 2 x 10^6 cells for 4x, starting from the 30th day after the irradiation with an interval of 15 days. Keep the infused rat in a warming blanket until it has recovered from the anesthesia.

5. Histology Analysis

5.1. Fix fresh aortic specimens in 10% formalin for 12 h. Rinse the tissue with phosphate-buffered solution (PBS) until the formalin is completely removed.

5.2. Dehydrate the aortic tissue in the automated vacuum tissue processor. Embed the tissue in fresh new paraffin and cut it into 5 µm cross sections.

5.3. Stain the sections with hematoxylin-eosin and Masson's trichrome to evaluate the vasculopathy.

5.4. Stain the sections by the standard avidin-biotin complex technique to measure the expression of myeloperoxidase (MPO) 16.

5.5. Obtain slide images under a digital slide scanner. Measure the thickness of the vascular wall by slide image analysis software. Normalize the intimal thickness to the full thickness of the vascular wall to obtain the relative value.

6. Fluorescent Staining

6.1. Mount fresh graft aorta in OCT compound and cut it into 5 µm cross sections.

6.2. Stain the nuclei with 4',6-diamidino-2-phenylindole (DAPI).

CAUTION: DAPI is mutagenic, and it should be handled with precaution.

6.3. Count GFP-labeled cells in the sections under a fluorescent microscope.

6.4. Calculate the average number of GFP-labeled cells per high-power field (HPF) from three random HPFs for each rat. Investigate eight rats from each group to obtain the average.

7. Real-time Quantitative Reverse Transcription Polymerase Chain Reaction

7.1. Total RNA extraction

7.1.1. Store fresh graft aorta tissue in liquid nitrogen immediately after it is harvested.

7.1.2. Weigh 5 mg of the aortic sample from each rat. Put the eight samples of the same group together as a biopsy specimen of 40 mg from the group. Homogenize the aortic tissue with a Dounce tissue grinder in an ice bath.

7.1.3. Add 1 mL of commercial trypsin solution to dissolve the tissue fragment and transfer the homogenate to a 2 mL centrifuge tube for centrifugation at 12,000 x *g* for 10 min at 4 °C.

7.1.4. Transfer the supernatant to a prechilled, fresh 2 mL centrifuge tube and keep it at room temperature for 5 min.

7.1.5. Add 0.2 mL of chloroform, vortex for 15 s, and leave it at room temperature for 3 min. Centrifuge at 12,000 x *g* for 15 min at 4 °C.

7.1.6. Transfer the aqueous phase (the top phase) to a fresh 2 mL centrifuge tube, being careful not to contaminate the solution with the other phases. Add 0.5 mL of isopropanol, mix them together, and incubate at room temperature for 10 min.

7.1.7. Centrifuge at 12,000 x *g* for 10 min at 4 °C. Discard the supernatant, wash the RNA pellet with 1 mL of 75% EtOH, and vortex for 15 s.

7.1.8. Centrifuge at 7,500 x *g* for 5 min at 4 °C. Remove the supernatant and allow the remaining EtOH to air-dry for 3 min.

7.1.9. Dissolve the RNA pellet in 20 µL of diethylpyrocarbonate-treated water and store it at -70 °C.

7.2. Reverse transcription

7.2.1. Prepare 10 µL of reaction mixture for each reaction. Mount each microtube with 2 µL of 5x Polymerase Master Mix, 2 µL of 100 ng/µL total RNA solution, and 6 µL of ultrapure water, as required to reach 10 µL.

7.2.2. Place the microtubes in a polymerase chain reaction (PCR) system. Start to run the program settings: 15 min at 37 °C, followed by 5 s at 85 °C.

7.2.3. Store the microtubes in an ice bath.

7.3. PCR analysis for cDNA samples

7.3.1. Prepare the primers of the following cytokines for cDNA template, according to previous studies[17,18]: tumor necrosis factor α (TNFα) forward primer 5'-CACGCTCTTCTGTCTACTGA-3' and reverse primer 5'-GGACTCCGTGATGTCTAAGT-3'; transforming growth factor β (TGFβ) forward primer 5'-CCTGGGCACCATCCATGA-3' and reverse primer 5'-CAGGTGTTGAGCCCTTTCCA-3'; interleukin 1β (IL-1β) forward primer 5'-GGGTTGAATCTATACCTGTCCTGTGT-3' and reverse

primer 5'-GACAAACCGCTTTTCCATCTTCT-3'; interleukin 2 (IL-2) forward primer 5'-CAGCTCGCATCCTGTGTTGCAC-3'and reverse primer 3'-GCTTTGACAGATGGCTATCCATC-3'.

7.3.2. Prepare 20 μL of reaction mixture for each reaction. Mount each microtube with 10 μL of 2x SYBR Premix Ex Taq II, 0.8 μL of forward primer, 0.8 μL of reverse primer, 0.4 μL of 50x ROX Reference Dye, 2 μL of cDNA solution, and 6 μL of ultrapure water, as required to reach 20 μL.

7.3.3. Place the microtubes in the PCR system. Start to run the program settings: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C.

7.3.4. Calculate the target RNA expression of each group by being normalized to that of β-actin and vehicle control using the comparative cycle threshold method.

8. Real-time Quantitative PCR

8.1. Genomic DNA preparation

8.1.1. Mount a fresh tube with a biopsy specimen of 10 mg from each group. Add lysis buffer A and proteinase K solution at 180 μL and 20 μL, respectively, vortex for 15 s, and incubate at 55 °C for 3 h to dissolve the tissue.

8.1.2. Add 200 μL of lysis buffer B, vortex for 15 s, and incubate at 70 °C for 10 min.

8.1.3. Add 200 μL of anhydrous ethanol and vortex for 15 s. Load the above mixture to a DNA purification column and centrifuge at 6,000 x *g* for 1 min.

8.1.4. Wash the column with 500 μL of buffer I and centrifuge at 6,000 x *g* for 1 min. Wash the column with 600 μL of buffer II and centrifuge at 18,000 x *g* for 2 min.

8.1.5. Load 200 μL of elution buffer to the column, keep it still at room temperature for 3 min, and centrifuge at 18,000 x *g* for 1 min. Place a fresh tube under the column to collect all elution which contains purified DNA.

8.2. PCR analysis for DNA samples

8.2.1. Prepare the primers for the Sry gene and for the housekeeping gene β-actin according to the reported sequence[19]: Sry forward primer 5'-GAGGCACAAGTTGGCTCAACA-3' and reverse primer 5'-CTCCTGCAAAAAGGGCCTTT-3'; β-actin forward primer 5'-CCATTGAACACGGCATTG-3' and reverse primer 5'-TACGACCAGAGGCATACA-3'.

8.2.2. Prepare 20 μL of reaction mixture for each reaction. Mount each microtube with 10 μL of 2x SYBR Green qPCR Master Mix, 1 μL of forward primer, 1 μL of reverse primer, 2 μL of 50 ng/μL DNA template, and 6 μL of ultrapure water, as required to reach 20 μL.

8.2.3. Place the microtubes in the PCR system. Use the PCR program settings: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C.

8.2.4. Calculate the Sry DNA levels by being normalized to that of β-actin using the comparative cycle threshold method.

9. Statistical Analysis

9.1. Express the data as the mean ± standard deviation. Make a group comparison by using the Mann–Whitney test. Consider a *p*-value of <0.05 to indicate statistical significance.

REPRESENTATIVE RESULTS:

On postoperative day 90, the segment of irradiated graft aorta was procured for histological analysis. The vascular injury consisted of intimal hyperplasia and vascular fibrosis, which resembled the histological changes of irradiated vessels reported previously[4]. The hyperplastic intima was formed by an accumulation of abundant spindle-shaped cells and extracellular matrix mixed with some degree of inflammatory cell infiltration. In Masson's trichrome stain, the amount of blue-stained collagen fiber was increased in all layers of the irradiated aorta, suggesting diffuse vascular fibrosis after irradiation. Moreover, a large number of MPO-positive cells gathered in the adventitia of the irradiated aorta. This indicated that severe oxidative stress occurred as it is commonly present in radiation injury[20]. In comparison, the histological response was almost the same between the RT-plus-TX and RT-only groups, while the aorta remained almost normal in the TX-only group as compared to the vehicle control. However, the groups with MSC treatment (the RT-plus-TX + MSC and RT-only + MSC groups) demonstrated a great histological relief when compared with the RT-plus-TX and RT-only groups. The thickness of intima was significantly decreased, and diffuse fibrosis and inflammatory cell infiltration were also attenuated (**Figure 1**). Notably, MSC infusion had a more favorable effect on intimal hyperplasia in the RT-plus-TX model than in the RT-only model since the RT-plus-TX + MSC group had a significantly lower average thickness of intima (**Figure 2**).

Next, the homogenate of the irradiated aorta was sent for PCR analysis of inflammatory cytokines, including TNF-α, TGF-β, IL-1β, and IL-2. All cytokines were significantly increased in both RT-plus-TX and RT-only groups as compared to the TX-only and vehicle control groups (**Figure 3**). This suggested a proinflammatory response to radiation injury, which is consistent with previous studies[20,21]. However, MSC infusion attenuated vascular inflammation since most of these cytokine levels were significantly decreased in the groups with MSC treatment. Notably, the RT-plus-TX + MSC group had a greater decrease of TGF-β level than the RT-only + MSC group.

Last, the homing of MSCs to the irradiated aorta was assessed by counting GFP-labeled cells under a fluorescent microscope. GFP-labeled cells were preferably engrafted into the intima layer at the average density of 3.30 cells/HPF in the RT-plus-TX + MSC group. In contrast, GFP-labeled cells were nearly invisible in the RT-only + MSC group. The result was supported by PCR analysis for the Sry gene. The RT-plus-TX + MSC group had a significantly higher level of Sry gene than the

RT-only + MSC group (**Figure 4**). Therefore, the homing of MSCs to the irradiated aorta was greatly improved by utilizing the RT-plus-TX model.

FIGURE AND TABLE LEGENDS:

Table 1: Animal groups and treatment protocols. The symbols of '+' and '-' represent whether the referred treatment was given or not, respectively.

Figure 1: Cross-sectional images of the abdominal aorta. Serial cross sections of the aorta were processed with hematoxylin-eosin stain (H&E), Masson's trichrome stain (Masson), or immunostain with antimyeloperoxidase antibody using a DAB substrate kit (MPO). The images represent the investigation of eight rats for each group. The scale bar = 100 μm.

Figure 2: Histological analysis of intimal hyperplasia. The relative intimal thickness was normalized to the full thickness of the vascular wall and expressed as a percentage. Eight rats were investigated for each group. Group comparison was performed with the Mann–Whitney test. * $p < 0.05$. The error bar = standard deviation.

Figure 3: Expression of proinflammatory cytokines in the aorta. The expression of (**A**) TNFα, (**B**) TGF β, (**C**) IL-2, and (**D**) IL-1β in study groups is illustrated. The cytokine levels were measured by real-time qualitative reverse transcription PCR and normalized to the vehicle control. The experiment was repeated three times for each group. (**E**) To which one of the study groups each bar referred to in the histogram is indicated. The group comparison was performed with the Mann–Whitney test. * $p < 0.05$. The error bar = standard deviation.

Figure 4: Migration of mesenchymal stem cells (MSCs) to the irradiated aorta. MSC migration was investigated by two techniques: fluorescent microscopy for tracing cells with a green fluorescent protein (GFP) label and PCR analysis for the sex determination region on the Y chromosome (Sry) specifically carried by transplanted MSCs. (**A**) The GFP-labelled cells were more frequently present in the intima of the RT-plus-TX model. The density of the GFP-labelled cells was expressed in the cell number per high-power field to quantify the MSC distribution in the irradiated aorta. (**B**) The RT-plus-TX model had a significant increase of GFP-labelled cell density when compared to the RT-only model. (**C**) The Sry level was approximately six-fold higher in the RT-plus-TX model than in the RT-only model. For each group, eight rats were investigated by fluorescent microscopy, and the PCR analysis was repeated three times. The group comparison was performed with the Mann–Whitney test. * $p < 0.05$. The error bar = standard deviation. The scale bar = 20 μm.

DISCUSSION:

The MSC-based therapy holds great promise for treatment of radiation injury. Many animal experiments have demonstrated that the transplantation of MSCs attenuated radiation injury by inhibiting inflammatory response and promoting tissue regeneration[12,22,23]. The homing of MSCs to injured tissue is the prerequisite for generating that effect[13]. However, only a few studies clearly have shown how many cells finally migrate and engraft into irradiated tissues[19,24].

Moreover, highly sensitive methods like PCR analysis for specific biomarkers of transplanted MSCs are preferred to quantify the MSC recruitment in many researches[14,19,24]. This suggests a very low population of homed MSCs like what was revealed in the RT-only model. In this model, the transplanted MSCs with GFP label were seldom present in the irradiated aorta. Although the poor engraftment of MSCs would be multifactorial, the animal model was intrinsically relevant. Despite the use of a beam-limiting device to avoid unnecessary tissue irradiation, the body area that was eventually irradiated not only included the abdominal aorta but also adjacent tissue, both of which were likely to release a damage signal to stimulate MSC migration[25-28]. Thus, circulating MSCs would also be distributed into the adjacent tissue, which was unintentionally subjected to radiation injury, yet form a larger compartment of MSC homing than the irradiated aorta alone. In that case, the fraction of MSCs recruited to the irradiated aorta would be greatly decreased. Conversely, if the aorta was exclusively subjected to radiation injury, more circulating MSCs would gather in the irradiated aorta. The theory is supported by an early study which found that local irradiation promotes the migration of MSCs to the irradiated field[14]. Therefore, this study introduced the RT-plus-TX model. In this model, the aorta of the irradiated rat was anatomically separated from adjacent tissue and transplanted to the healthy counterpart. Although the irradiated vascular graft developed almost the same histological changes as what was seen in the RT-only model, a significant increase of transplanted MSCs with GFP label and Sry gene was found in the vascular graft. The following calculation predicted the relative changes of the MSC density in the irradiated aorta after the separation of the aorta from adjacent tissue like in the RT-plus-TX model.

In the RT-only model, the volume of irradiated tissue ($V_{RT\text{-}only}$) was calculated by multiplying the length (L), width (W), and depth (D) of the body compartment exposed to radiation. The length and width were determined by the square-shaped irradiation field of 3 x 3 cm, and the depth was the thickness of the rat posterior abdominal wall, approximately 1.5 cm on average. The result is shown as follows.

$V_{RT\text{-}only}$ = 3 cm (*L*) × 3cm (*W*) × 1.5 cm (*D*) = 13.5 cm^3

In the RT-plus-TX model, the aortic graft was the only tissue with irradiation. The graft was shaped like a cylinder with a height (H) of 1.5 cm and a cycle base area of 1 mm in diameter (A). The formula to calculate the irradiated volume ($V_{RT\text{-}plus\text{-}TX}$) is shown as follows.

$V_{RT\text{-}plus\text{-}TX}$ = 1.5 cm (*H*) × π × (0.1 cm (*A*)/2)^2 = 0.012 cm^3

If migrating MSCs are evenly distributed in the irradiated tissue, and if the number of migrating MSCs is constant (*i.e.*, the sum of infused MSCs possessing the high capacity of migration is not different between the RT-only + MSC and RT-plus-TX + MSC groups), then the MSC density of irradiated tissue is inversely proportional to the volume of irradiated tissue. Therefore, the MSC density (DEN) was calculated from the following formula.

$DEN_{RT\text{-}plus\text{-}TX}$ / $DEN_{RT\text{-}only}$ = $V_{RT\text{-}only}$ / $V_{RT\text{-}plus\text{-}TX}$ = 13.5 cm^3 / 0.0118 cm^3 = 1.14 x 10^3

As a result, the irradiated aortas of the RT-plus-TX+ MSC group were estimated to have approximately a 1,000x higher MSC density than those of the RT-only+ MSC group. In that case, the density of GFP-labeled cells in the RT-only + MSC group was anticipated to be 0.0029 (3.30 / 1.14 x 10^3) cells/HPF, which suggested a fairly low incidence of detecting GFP-labeled cells under fluorescent microscopy. The estimate was supportive of what was observed in this experiment, although many factors, like whether migrating MSCs were evenly distributed between the aorta and adjacent tissue, were neglected. Intriguingly, a previous study revealed that MSCs were preferably homed to the viscera, skin, and muscle, but not aorta, after local irradiation[14]. In other words, the aorta was not the favorable destination for MSCs as compared to adjacent tissue and organs if isodose irradiation was given. Therefore, the sequestration of the irradiated aorta was presumably helpful to diminish the preference of MSC recruitment to adjacent tissue. Generally, the RT-plus-TX model supported the theory that simultaneously irradiated adjacent tissue interfered with the gathering of MSCs to the irradiated aorta. The sequestration of irradiated rat aorta by transplantation to a healthy counterpart was an effective way to improve the MSC local recruitment.

However, the RT-plus-TX model had some drawbacks. First, this model is suitable for the study of large vessels but not the microvascular system. Radiation vasculitis is morphologically different depending on the size of the vessels. When compared with large vessels, capillary vessels are prone to rupture and dilate and form thrombus after irradiation[29]. Such pathological features were not present in the RT-plus-TX model. Moreover, the method to sequester irradiated vessels as described in this model is not applicable to microvasculature, since the transplantation of capillary vessels alone is technically difficult. Second, the RT-plus-TX model is not easy to use, especially for beginners who have had no training for microsurgery. The major obstacle is to complete aortic anastomosis in a short time without any serious complication like bleeding and thrombosis. Therefore, the technique of cyanoacrylate-assisted vascular anastomosis was introduced to simplify the procedure of aorta transplantation and improve the success rate. The use of cyanoacrylate is safe and effective enough, as reported in many studies[30-32]. Third, some might argue that the RT-plus-TX model is not reliable because surgical trauma, such as ischemia-reperfusion injury, will accelerate the progression of radiation vasculitis. Admittedly, surgical trauma is unavoidable in this model, given that aorta transplantation is indispensable to sequester irradiated aorta. But most follow-up effects of surgical trauma were temporary, being initiated shortly after the operation and regressing within 1 month according to a previous study[32]. Moreover, the interference of surgical trauma was well controlled by setting up the RT-only group which served as sham surgery control. Consequently, the RT-plus-TX group shared similar pathological features of radiation vasculitis as the RT-only group. Last, the contribution of immune rejection was eliminated by the transplantation between F344 inbred rats which possessed minimal genetic differences within the strain.

Generally, the RT-plus-TX model promotes MSC accumulation in irradiated vessels by separating an irradiated vascular segment from adjacent tissue. This model is preferred in the study of MSC-based therapy for radiation vasculitis when the evaluation of MSC homing is demanding.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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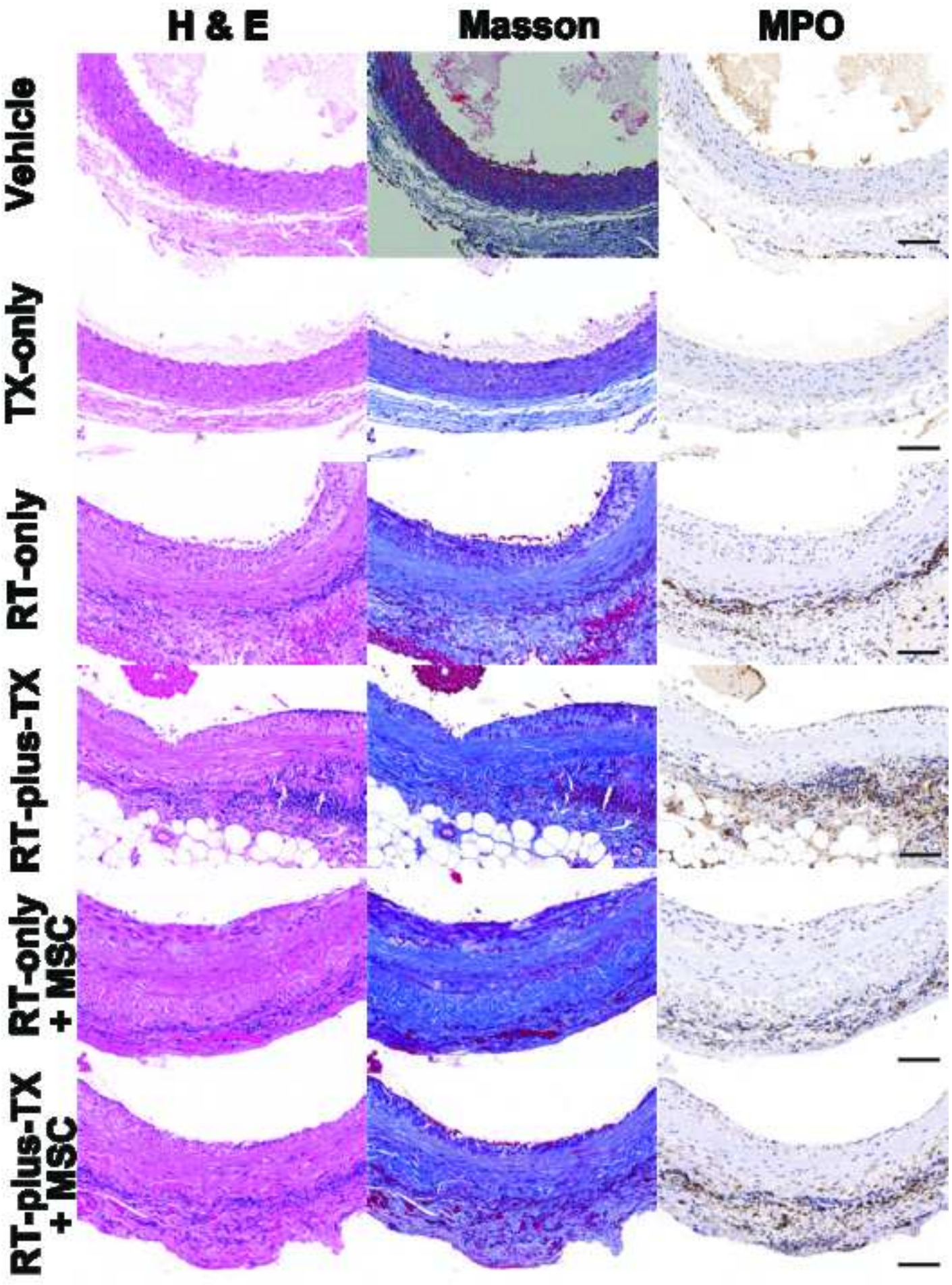

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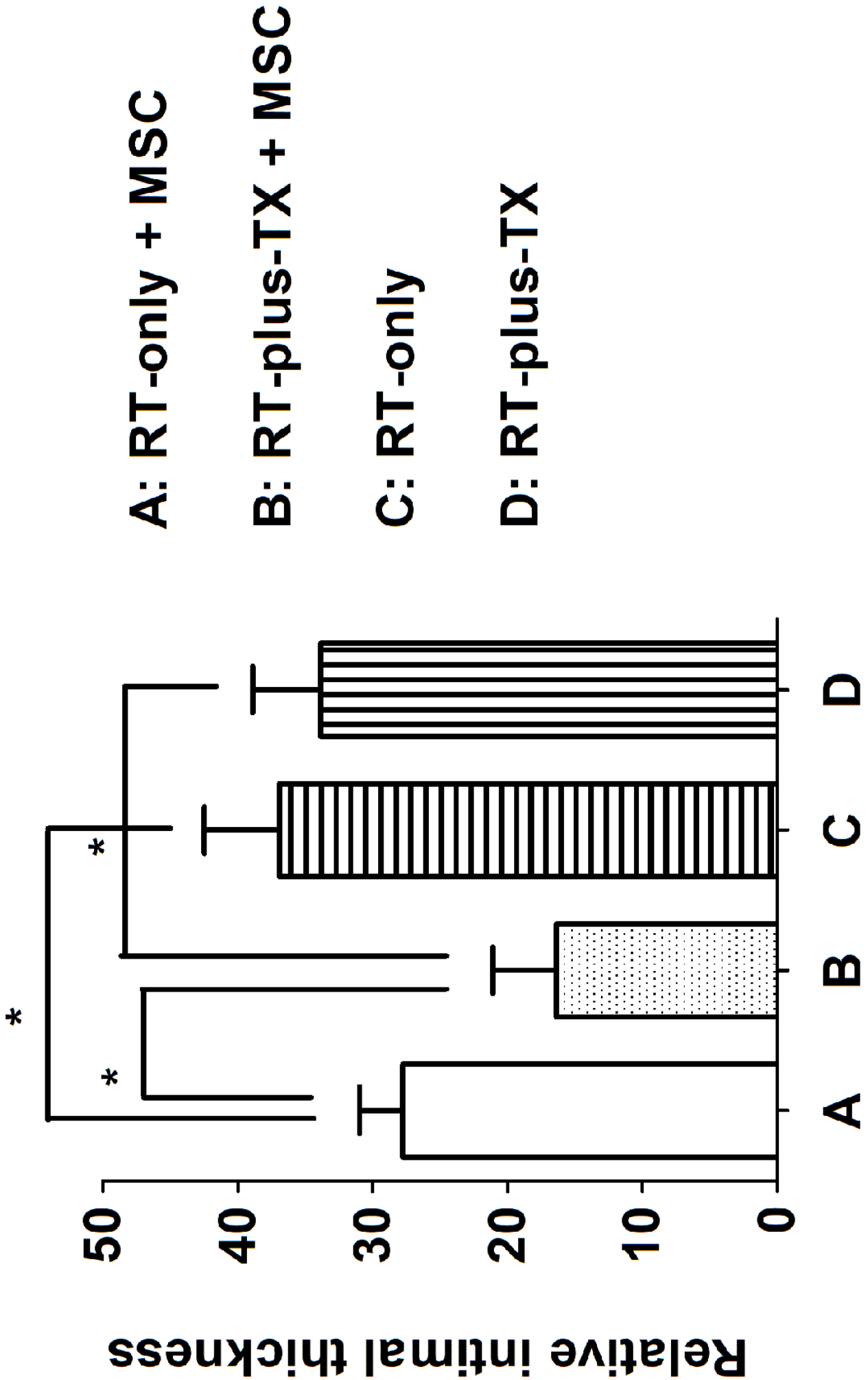

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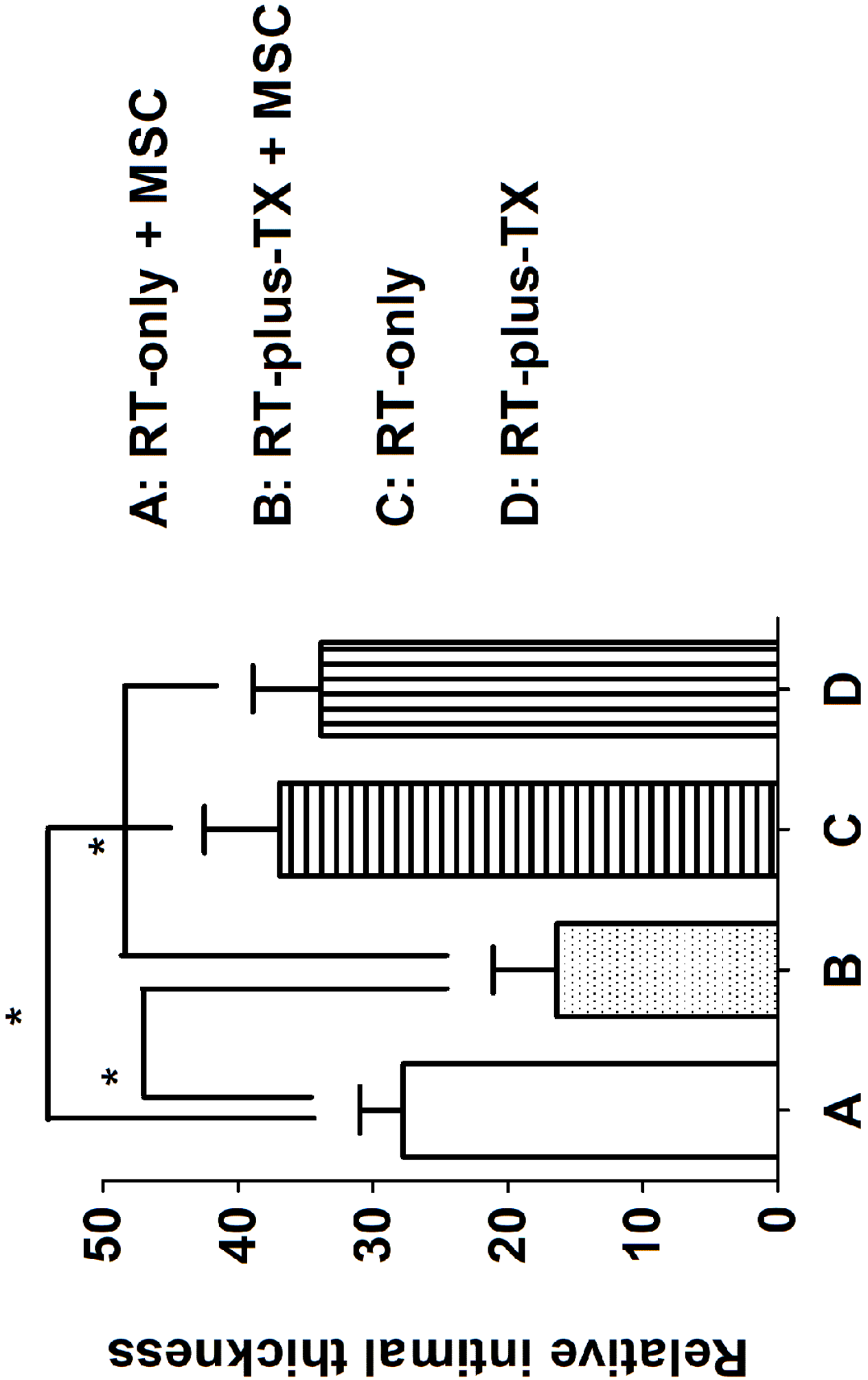

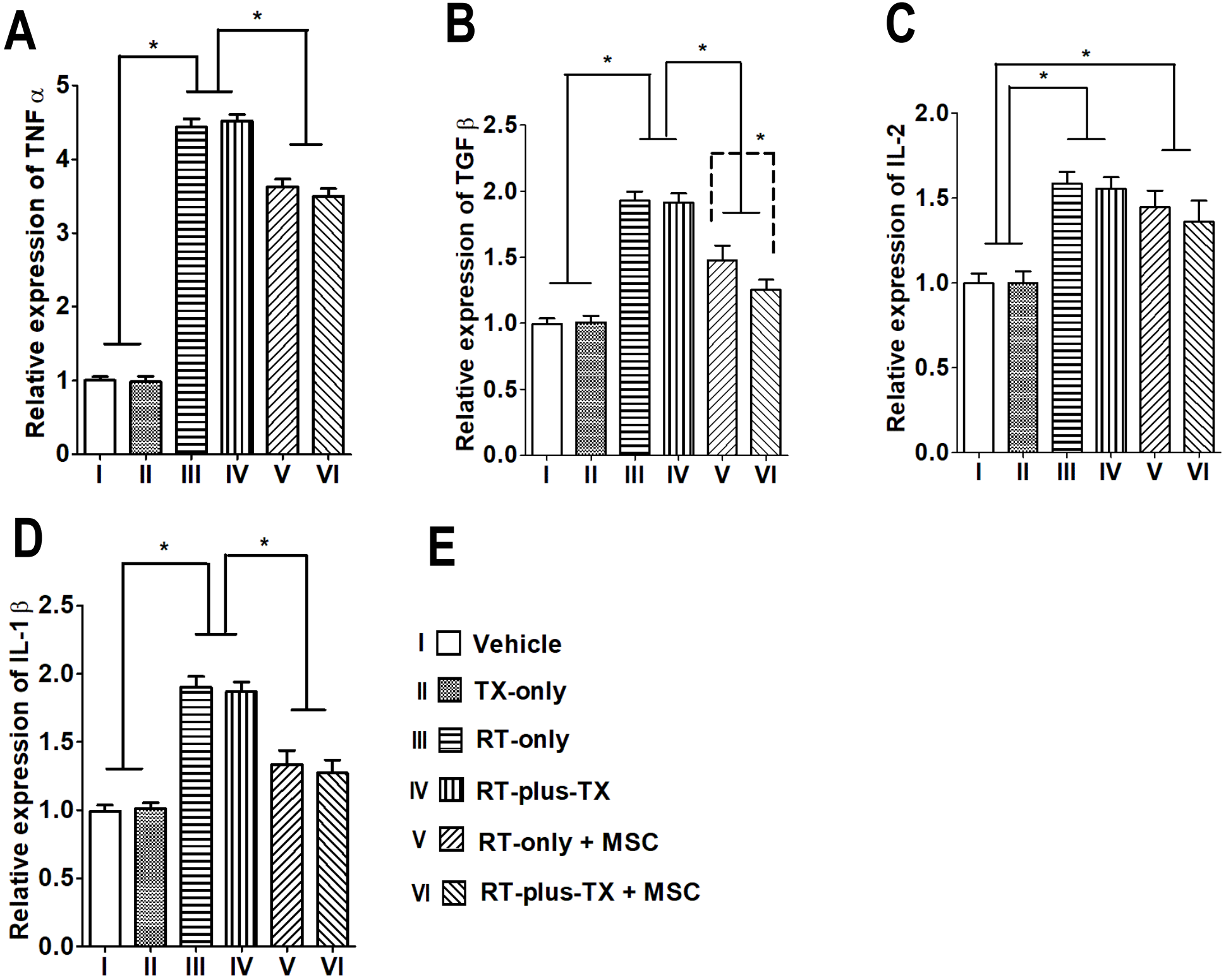

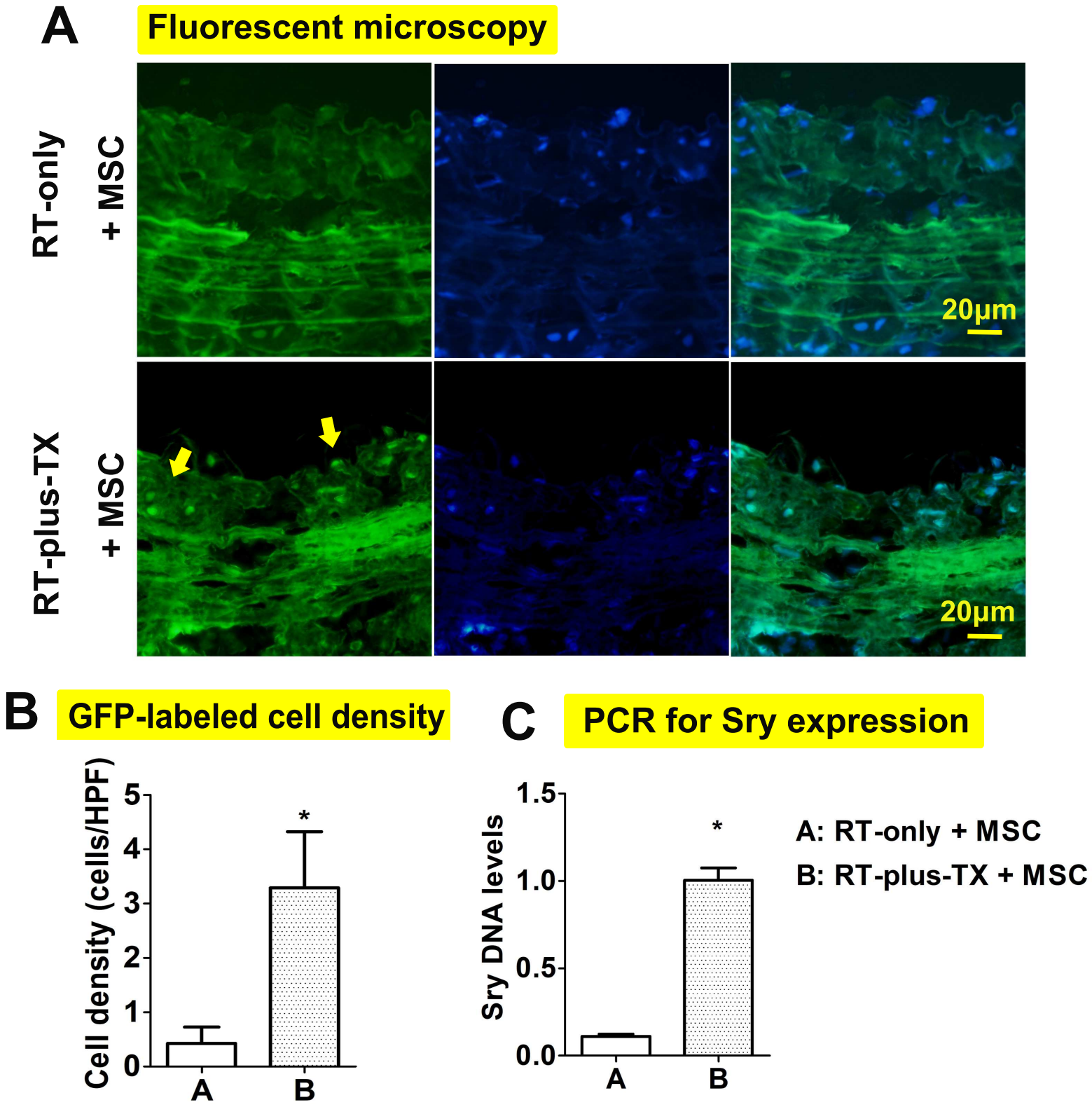

Table 1

Group	n	Radiation	Aorta Transplantation	MSC infusion
Vehicle control	8	–	–	–
RT-only	8	+	–	–
TX-only	8	–	+	–
RT-plus-TX	8	+	+	–
RT-only+MSC	8	+	–	+
RT-plus-TX+MSC	8	+	+	+

Table 2

Cell markers	CD90	CD44	CD29	CD34	CD45
Fold changes after transfection	0.997±0. 078	0.983±0.051	0.980±0.062	0.960±0.061	1.057±0.040

CD11b/c
0.953±0.031

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10% Chloral hydrate solution	Leagene Biotechnology, China		
10% Fetal bovine serum	Thermo Fisher Scientific, USA	12664025	
2 μL pipette with tips	Eppendorf, Germany		
2×SYBR Premix Ex Taq II	Takara, China	RR820L	
3-0 Polyglactin suture	Ethicon, USA		
4′,6-diamidino-2-phenylindole	Thermo Fisher Scientific, USA	D1306	
5× PrimeScript RT Master Mix	Takara, China	RR036	
9-0 Nylon suture	Jinhuan Medical Products, China		
Automated Vacuum Tissue Proc	Leica Biosystems, Germany	ASP6025	
Biological irradiator	Radsource, USA	RS2000 pro	
Biotinylated anti-rabbit secondary antibody	Agilent Technologies, China	E0353	
Buffer Ⅰ	Beyotime, China	D0063-3	
Buffer Ⅱ	Beyotime, China	D0063-4	
Colibri retractor	RWD Life Science, China	R22029-03	
Digital slide scanner	Pannoramic Desk	3DHISTECH, Hungary	
DNA purification column	Beyotime, China	D0063-7	
Dulbecco’s modified Eagle’s medium	Thermo Fisher Scientific, USA	11885092	
Elution buffer	Beyotime, China	D0063-5	
Female F344 rats	Vital River Laboratory Animal Technology, China		12 weeks of age
Forceps	RWD Life Science, China	F22003-09	
Genomic DNA mini preparation	Beyotime, China	D0063	

Genomic DNA mini preparation kit with spin column	Beyotime, China	D0063
Heparin	Qianhong Bio-pharm, China	
Horseradish peroxidase-conjugated streptavidin	Agilent Technologies, China	P0397
Interleukin 1β primer	Sangon Biotech, China	
Interleukin 2 primer	Sangon Biotech, China	
Lentivirus encoding green fluorescent protein gene	Genechem, China	
Lysis buffer A	Beyotime, China	D0063-1
Lysis buffer B	Beyotime, China	D0063-2
Male F344 bone marrow mesenchymal stem cells	Cyagen Biosciences, China	RAFMX-01001
Microclamps	Jinzhong Medical Instrument, China	W40130
Microneedle holder	Jinzhong Medical Instrument, China	WBD020
Microscissors	Jinzhong Medical Instrument, China	WAA010
Microtweezers	Jinzhong Medical Instrument, China	WA3010
Needle holder	RWD Life Science, China	F31026-13
Normal saline	Baxter, China	
PCR system for step 7	Thermo Fisher, USA	Applied Biosystem 7500
PCR system for step 8	Roche, USA	LightCycler 480
Polyclonal MPO antibody	Agilent Technologies, China	A0398
Primer for sex determination region on the Y chromosome	Sangon Biotech, China	
Proteinase K solution	Beyotime, China	D0063-6
Scissors	RWD Life Science, China	S13052-12
Slide image analysis software	Panoramic viewer	3DHISTECH, Hungary

Stereo microscope	Jiangnan Novel Optics, China	JSZ6
Tissue adhesive	B. Braun, Germany	Histoacryl blue
Transforming growth factor β primer	Sangon Biotech, China	
Trizol solution	Beyotime, China	R0016
Tumor necrosis factor α primer	Sangon Biotech, China	
β-actin	Sangon Biotech, China	

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Line 206: How are the intimal thickness and full thickness measured?

Necessary revision is made.

Line 219: Do you mean liquid nitrogen?

Yes, necessary revision is made accordingly.

Line 267: Please provide composition of lysis buffer A. If it is purchased, please cite the Table of Materials.

Yes, it was purchased. More information is provided in JOVE Materials.

9. Lines 116-121: Please split into two steps.

Necessary revision is made.

10. Lines 129-140: The Protocol should contain only action items that direct the reader to do something. Please move the equipment information to the Materials Table.

Necessary revision is made.

11. Lines 296-298: Please write the text in the imperative tense.

Necessary revision is made.

12. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

It is done.

13. Please include single-line spaces between all paragraphs, headings, steps, etc.

It is done.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

It is done. The part with grey background include the essential steps of protocol for video recording.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

It is done.

16. Please include all relevant details that are required to perform the step in the highlighting.

For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

It is done.

17. Table 1 is missing from the submission. Please upload Table 1 to your Editorial Manager account as an .xls or .xlsx file.

Table1 was uploaded to the submission system at the beginning. But I do not know why it was not presented to you. Anyway, I do it again. Please find attached xlsx file.

18. Figures 2-4: Please define error bars in the figure legend.

It is done.

19. Figures 3 and 4: Please briefly describe different panels of the figure.

It is done.

20. Figure 4: Please define scale bars in the figure legend.

It is done.

21. Discussion: Please discuss critical steps within the protocol.

It has been discussed in the paragraphs from line 428 to 450.

22. References: Please do not abbreviate journal titles.

Full journal title is provided.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This study investigated the homing of MSCs in the model of irradiation vasculitis after their intravenous infusion. Thus six study groups were employed including that of irradiation plus MSC infusion and irradiation then transplantation of irradiated aorta to a healthy recipient rat followed by MSC infusion, together with other needed control groups. The study results showed that homing of MSCs was significantly higher in the transplanted irradiated aorta and thus showed better histological repair and reversed inflammatory response.

Major Concerns:

1- Table 1 is not included in the manuscript which made it hard to assume the study groups , the reviewer assumed such information from the given figures. Table 1 should be added and reviewed

Table1 was uploaded to the submission system at the beginning. But I do not know why it was not presented to you. Anyway, I do it again. Please find attached xlsx file.

2- Some details are missing in the MSC infusion protocol , these details are mandatory for others to follow your protocol of work and they include :

-- DMEM basal media used , was it high or low glucose , with or without L- glutamine

Low-glucose DMEM with L-glutamin was used in the study.

-- The passage of MSC cells used for the infusion

Passage 3 of MSC cells after viral transfection was used for intravenous infusion.

-- What MSCs characterized method was used before the infusion of these cells

The expression of MSC markers was checked by realtime-PCR. As a result, there were no significant changes in the expression of positive MSC makers like CD90, CD44, CD29, and negative markers CD34, CD45, CD11b/c. The relevant data are demonstrated in Table 2.

-- Duration of infusion of cells through the tail vein

The single dose of 2×10^6 cells was prepared in 0.4ml serum-free medium and infused to each rat via tail vein for 15 sec.

Minor Concerns:

1- Authors should mention in animals section that male rats were used for bone marrow isolation and transfusion in the female rats, for the purpose of tracking the cells in the transplanted aorta using the SYR gene as its first mention is in the results section

The changes are made according to the suggestion.

2- It is clear that the aim of this study is evaluating the MSC homing for irradiation vasculitis thus irradiated vessels were sequestered from adjacent tissue and transplanted in recipient animal representing the RT- plus- Tx model. Showing the promising study results, how do the authors think such model could be translated clinically so that avoid scattering of transplanted cells to adjacent tissue?

Our study was based on the previous research showing that local irradiation enhanced MSC recruitment to the field of radiation. Sequestration of irradiated vessels from adjacent tissue was an effective way to make it sure that radiation energy was exclusively delivered to target vessels. As a result, MSC recruitment was improved by our attempt. Our study provided further evidence to the previous research and suggested the advantage of local irradiation. Based on the theory, many methods of delivering local irradiation like intensity modulated radiation therapy are recommended not only to reduce the risk of injury to unintentionally irradiated organs but to improve the effect of MSC-based therapy.

Reviewer #2:

Manuscript Summary:

The authors have developed a model of recapitulates aspects of radiotherapy induced vasculitis. In an effort to limit confounding factors, the authors have developed a model in which the abdominal aorta of a donor rat is extracted and subsequently irradiated prior to transplantation into a recipient rat. Following transplantation the animals are treated with syngenetic, GFP labeled MCSs. Animals are then euthanized at Day 90 (60 days post initial treatment administration) for histological analysis. This is an interesting method that models key aspects of radiotherapy induced soft tissue damage that allows for the investigation of both MSC homing and MSCs' ability to modulate RT-induced tissue remodeling.

Major Concerns:

None

Minor Concerns:

The rationale for the use of a non-parametric rank test statistical analysis (Mann-Whitney) is

not clear to this reviewer, as there the samples were all garnered from a similar system so there would be an expectation that their respective distribution would be the same, in addition to the same samples sizes were used for each treatment group.

Although the method of data collecting for each variable was same between study groups, the variable did not necessarily obey normal distribution which was considered as the criteria of parameter analysis. For example, the relative intimal thickness (the percentage of thickness of intima to full vascular wall) and the cell number per high power filed were more likely to obey Poisson distribution than normal distribution. In that case, Mann-Whitney test was more suitable than parameter analysis to be used in our data analysis.