

Original Article

A refined mouse hind limb model of secondary lymphedema

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Abstract: Background: This study aimed at establishing a more stable and persistent mouse lymphedema model through modification of radiation dose and administrating timing. Methods: Mice lymphedema models were created by using a combination of lymph node removal and radiation treatment. The experiment groups received the following treatments at varied time points: "surgery before radiation" group (S+R group; n = 12), 4.5 Gy at 2 weeks after surgical treatment; "radiation treatment before surgery" group (R+S group; n = 12), 4.5 Gy at 3 days before surgery; and "radiation before and after surgery" group (r+S+r group; n = 12), 2.25 Gy at both 3 days before and 2 weeks after surgery. Volume change was compared by using the water replacement method. Lymphatic vessel distribution was visualized by in vivo lymphatic imaging with fluorescence nanogel. Immunohistochemistry staining was performed to show superficial lymphatic vessels. Lymphatic vessel density was compared. Results: All the treated mice developed hind limb lymphedema. The r+S+r group showed the most stable hind limb lymphedema during our observation period of 6 months, with lower mortality and morbidity rates. At 6 months post operation, lymphatic imaging revealed superficial lymphatic network for all the groups. The number of lymphatic vessels passing through the surgical scar was significantly higher ($P < 0.05$) in the R+S (n = 9) and S+R groups (n = 9) than in the r+S+r group (n = 10). Immunohistochemistry revealed a significantly higher number of lymphatic vessels distal to the treated area in the r+S+r group than in control and R+S groups ($P < 0.05$). Conclusion: A radiation dose of 2.25 Gy administered 3 days before surgery and 2 weeks after surgery successfully enhanced the stability of the mice hind limb lymphedema model.

Keywords: Lymphedema, animal model, lymphatic vessels

Introduction

Lymphedema is the accumulation of excess lymphatic fluid in interstitial spaces caused by obstruction of the lymphatic system [1]. Secondary lymphedema commonly occurs after damage to the lymphatic vessels or surgical removal of lymph nodes during breast cancer and pelvic malignancy surgeries [2-7]. Despite the substantial advances in surgical strategies for tumors, the therapeutic options for secondary lymphedema are limited, even at present [6, 8-10]. Investigations and treatment of the disabling disease are urgently needed, but they are seriously hindered by incomplete clinical therapeutic approaches and a deficiency in fundamental research studies, which mainly result from lack of a stable lymphedema animal model [10, 11].

A reliable and inexpensive method of reproducing lymphedema in a small animal as an experimental counterpart of the human disease would be of great significance for developing effective therapeutic approaches. Many attempts have been made to establish an animal lymphedema model in the last decades [12-19], but none of these have been able to reliably develop a sustained chronic effect similar to that found in human secondary lymphedema. However, these studies demonstrated that surgery alone is insufficient to induce reliable and sustained lymphedema [13, 16, 20]. Associated problems mainly included procedural complexity, protracted time before stabilization of the chronic lymphedema, and uncontrollable recovery from lymphatic regeneration. At the same time, when relying on radiation alone to create animal models, a previous study failed to induce

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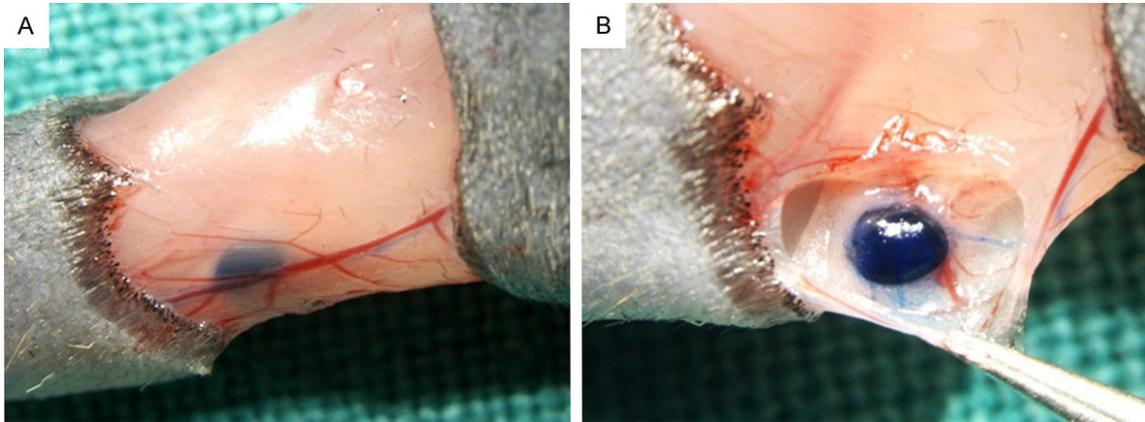


Figure 1. Formation of lymphedema in the hind limbs of C57 mice. A. Staining of the popliteal lymph nodes. B. Removal of the lymphatic tissue and preservation of the neurovascular tissues.

overt lymphedema with low-dose radiation [17], and high-dose (9 Gy) radiation significantly increased the mortality rate caused by infection in our previous study (data not shown). However, the combination of surgery and radiation has been demonstrated to be more effective in producing lymphedema models than either surgery or radiation alone [13, 16, 20]. According to previous reports, two combination strategies have been proposed for establishing a lymphedema model, namely radiation before surgery and radiation after surgery [6, 9]. Lee-Donaldson reported that both strategies could prolong the edematous period to 60 days, further confirming the superior efficacy of surgery combined with radiation in producing a more reliable lymphedema model [16]. Unfortunately, a radiation dose of 4.5 Gy and an interval of 3 days between surgery and radiation resulted in a high mortality rate (9%-13%) and limb necrosis. Although he used more-radical operative procedures to eliminate lymphatic vessels, lymphedema did not last for 1 week, which is far less than the 8 weeks of swelling induced by surgery combined with radiation. However, the limb necrosis and high mortality rate remained high in his study [6]. Taken together, the need for a stable and sustained lymphedema model with low mortality and morbidity rates has become an overwhelming issue for lymphedema therapeutic research.

To address the above-mentioned problems, in the present study, we designed 3 combinations of radiation and surgery and used different radiation doses to determine the optimal radi-

ation condition for developing a stable and sustained lymphedema animal model that could be applied for further biological and therapeutic research.

Methods and materials

Animals

Thirty-six male C57 mice aged 8 weeks (acquired from the Animal Institute of Chinese Academy of Science, Shanghai, China) were maintained under specific pathogen-free conditions and evenly divided into three groups as follows: the SR group, treated with surgery followed by radiation of 4.5 Gy; the RS group, treated with radiation of 4.5 Gy followed by surgery; and the r+S+r group, treated with radiation of 2.25 Gy before and after surgery. All the experiments that used mice were performed in accordance with the Chinese National Institutes of Health guidelines for the care and use of laboratory research animals.

Surgery

For lymphatic mapping, to clearly localize deep lymphatics, we adopted the fluorescent dextran-poly (acrylic acid) nanogel (FDNG) mentioned in our previous report [21], which can be used as a biosafe in vivo tracing agent for imaging lymphatics in detail, without causing harm to animals. All the animals were anesthetized by using an intraperitoneal injection of 5% chloral hydrate. The FDNG nano probe (20 μ g of samples in 20 μ L of water) was intradermally

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Table 1. General condition of experimental animals

	Treatment	Death	Limb necrosis	Mortality rate	Morbidity rate
S+R	Surgery+4.5 Gy	3	2	25%	17%
R+S	4.5 Gy+Surgery	3	1	25%	9%
r+S+r	2.25 Gy+Surgery+2.25 Gy	1	0	9%	0

injected in the hind paw of a mouse and imaged for in vivo migration of the FDNG (5-AF) probe under a fluorescent optical imaging system.

After the deep lymphatics were exactly visualized, methylene blue was injected subcutaneously in the right footpad to enable operation under white light. A circumferential incision was made on the right hind limb, 1 cm above the knee, and a 1-cm circumferential strip of skin and subcutaneous tissue was resected. Neurovascular bundle and muscles, and tendons were carefully preserved through operation. Stained lymphatic and circumferential margins were electrocauterized, and popliteal lymph nodes were removed under microscopy (**Figure 1**). Erythromycin was spread on the wound area for infection prevention.

Radiation treatment

The inguinal areas of the mice were exposed to Cs139 radiation. The size of exposure field was 1 · 1 cm², and the rest of the mouse body was shielded from radiation by using lead blocks. In accordance with the radiation dose in previous studies, a 4.5-Gy radiation treatment was administered to the S+R group 2 weeks after surgery, and the R+S group received the same radiation dose 3 days prior to surgery. The r+S+r group received a 2.25-Gy radiation (Cs119) dose at 2 time points as follows: at 3 days prior to surgery and at 2 weeks after surgery. Treatment was conducted unilaterally, with the untreated limbs used as controls.

Water displacement volumetric analysis

To measure the volume change in the affected limbs, we used the water displacement method. The limbs were placed inside 10-ml cylinders fully filled with saline up to the most proximal margin of the excised wound. The volume of water used to refill the cylinder indicated the limb volume. The unaffected limb was also measured as the corresponding control. Measurement was performed weekly for 6

months. Each measurement was performed 3 times, and a mean value was obtained.

Fluorescence microscopy for lymphatic pattern imaging

To investigate the distribution of lymphangiogenesis in the hind limbs, we obtained fluorescence microscopy photos 24 weeks after surgery. Under anesthesia, FDNG was injected into the hind paws for in vivo lymphatic imaging, with gentle massage of the footpad for better distribution. Photos were obtained immediately by using a Leica microscopic camera for examination of the uptake of fluorescence and lymphatic pattern.

Immunohistochemical examination

To further analyze the quantity and morphology of newly formed lymphatic vessels, all the mice were killed 24 weeks after surgical treatment. The skin and muscle specimens from the hind limbs were obtained for immunohistochemical staining. Tissues were fixed in 4% formalin, embedded in paraffin, and sectioned into 5- to 7- μ m slices. After deparaffinization, rehydration, and blocking, the tissues were incubated with primary antibody for LYVE-1 (1:400, Angiobio, USA) at 4°C overnight. The next day, the slides were incubated with horseradish peroxidase goat anti-mouse antibody (1:200, Google Biotech, China) for half an hour. Diaminobenzidine (DAB) staining was performed with the REAL Envision Detection System, Peroxidase/DAB+, and Rabbit/Mouse (Dako), according to the manufacturers' instructions, sequentially conjugating secondary goat antigen and staining for DAB. LYVE-1-positive tubular structures were counted from 4 random high-power microscopic fields by using Image Pro Plus 4.5 (Cybernetics Inc.). The mean value was calculated.

Statistical analyses

All data are presented as means \pm SE. A two-way ANOVA with post hoc test using Tukey's method was performed in comparison of the change of limb volume; a one-way analysis of variance with the post hoc Tukey test was performed in comparison of lymphatic vessel density and skin thickness in immunohistochemistry staining. IBM SPSS Statistics 20 (IBM) was

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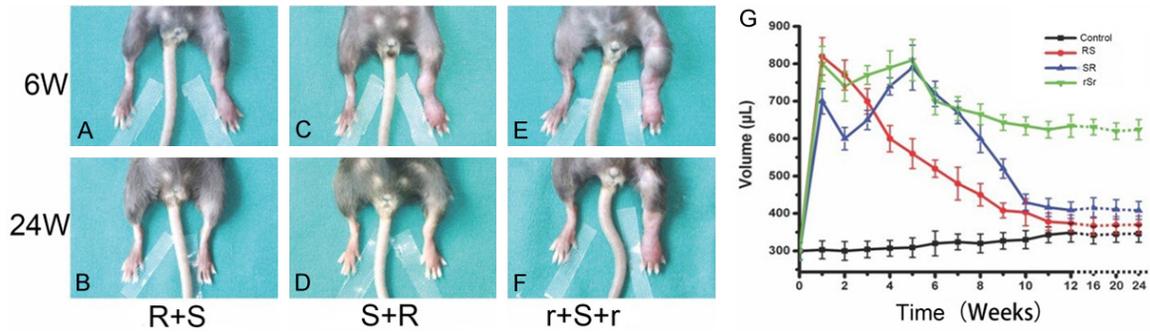


Figure 2. Changes of the edematous hind limbs after surgery. A, C, E. Edematous state at 6 weeks after surgery. B, D, F. Edematous state at 24 weeks after surgery. G. Volume measurements for the R+S, S+R and r+S+r groups at different time points. Unaffected limbs used as controls are also shown.

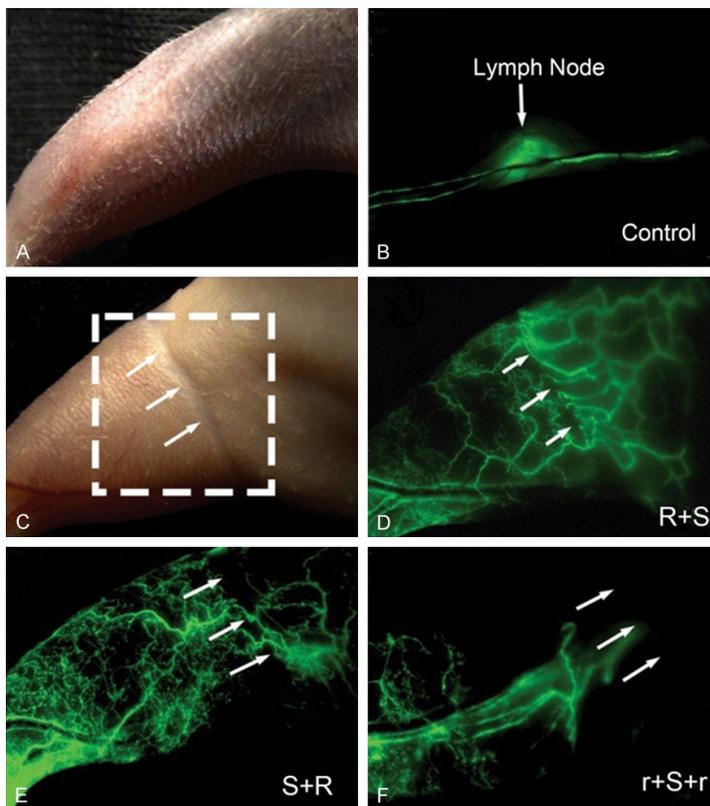


Figure 3. Lymphatic mapping of hind limbs in vivo 24 weeks after surgery. A. Normal hind limb under white light. B. Normal hind limb under fluorescent light after injection of FDNG, which is absorbed by the deep lymphatic vessels, and the lymph node can be clearly visualized. C. Lymphedemic hind limb under white light. The dashed area indicates the radiated site, and the arrows indicate the scar caused by the surgical treatment. D and E. Large number of micro lymphatic vessels forming in the skin and passing through the circular incision scar tissue in the R+S and S+R groups. The arrow indicates the lymphatic vessels passing through the scar and forming a reconnection. F. Smaller micro lymphatic formation, without reconnection passing through the incision scar, in the rSr group. The white arrows indicate the scar tissue. The average number of lymphatic vessels in the dashed area in the R+S group (n = 9) and S+R group (n = 9) are significantly higher than that in the r+S+r group (n = 11; $P < 0.05$).

used for analysis, and differences were considered statistically significant when the P value was < 0.05 .

Results

General animal condition

During the observation period, all the mice developed an acute lymphedema immediately after surgical treatment. In the r+S+r group, repeated low-dose radiation treatment was well tolerated, but one mouse died of anesthesia. In the S+R group, three mice died of infection and five mice developed limb necrosis. In R+S group, three mice died of infection and four mice developed limb necrosis (Table 1).

Assessment of the animal model for lymphedema formation

The degree of lymphedema in our animal model was measured by performing a volumetric analysis by using a water displacement method. Within 1 week after surgery, the R+S and r+S+r groups showed significant higher increases than the S+R group. After 1 week, the volume in all the groups declined, with the volume declining slower in the R+S and r+S+r groups than in the S+R group. At the second week after surgery, radiation treatment was adminis-

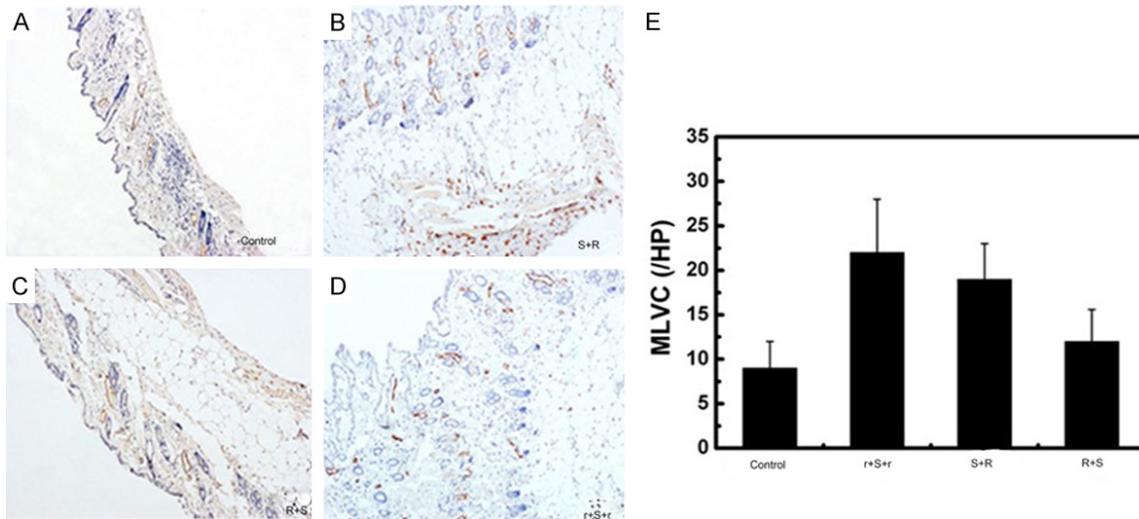


Figure 4. Immunohistochemical staining of the lymphatic vessels. Tissue specimens were harvested from the distal part of the treated area. A. An unaffected hind limb used as a control. B-D. The skin specimen from the treated groups is significantly thicker than that from the control group ($P < 0.05$), with more LYVE-1-positive vessels. E. LYVE-1-positive vessel in the r+S+r group showing significantly higher density than control and R+S groups.

tered to the mice in the S+R group at 4.5 Gy and to those in the r+S+r group, at 2.25 Gy. Then, the volumes in the two groups continued increasing until the sixth week (**Figure 2A, 2C and 2E**). By contrast, the R+S group showed continued volume decrease due to the absence of radiation at the second week. Six weeks later, the volume in all the groups declined. The volumes in the R+S and S+R groups returned to the same level as that in the control group after 10 and 12 weeks, respectively. As regard to the r+S+r group, no obvious change was observed after 8 weeks and the edematous state remained until the end of our observation period (**Figure 2B, 2D and 2F**). The continuous volumetric change in 24 weeks is presented in **Figure 2G**.

Imaging of lymphatic vessels in the lymphedemic hind limbs

To investigate the distribution of lymphatic vessels in the hind limbs, FDNG was used as a tracer for lymphatic vessel imaging at 6 months after surgery [21]. The examination revealed that the skin of the affected limbs showed highly dense and curved lymphatic vessels that formed a branched network (**Figure 3A-D**) as compared with the normal limbs (**Figure 3E and 3F**), while deep lymphatics could not be visualized. In the R+S and S+R groups, a large number of micro lymphatic vessels were passing

through the incision scar tissue and formed a reconnection (**Figure 3B and 3C**). In comparison, in the r+S+r group, few lymphatics regenerated and failed to reconnect (**Figure 3D**). The results indicated that the obstructed deep lymphatic vessels were difficult to reconnect owing to the weak regeneration capacity. The reconstructed collateral circulation and regeneration of the superficial micro lymphatic vessels played a dominating role in lymphatic compensation.

Evaluation of lymphangiogenesis of lymphedema hind limbs

To further analyze the quantity and morphological changes of the lymphatic vessels, the specimens from the lymphedemic hind limbs were immunohistochemically stained. The number of lymphatic vessels in the r+S+r group was lower than that in the R+S and S+R groups (**Figure 4A-D**). Morphological examination of the lymphatic vessels in the treated group revealed thin-walled and dilated vessels with curvature (**Figure 4A-C**) as compared with those in the untreated limbs (**Figure 4D**). Statistical analysis further confirmed that the number of skin lymphatic vessels in the r+S+r group was significantly lower than those in control and R+S groups (**Figure 4E**), which suggests that the repeated radiation suppressed the lymphatic regeneration more effectively.

Discussion

In the present study, we introduced a potent means of creating a stable lymphedema animal model. For the first time, a lymphedema model maintained an edematous state for a period of more than 6 months, which is markedly longer than that of any previously reported models. A more important finding is that the mortality and morbidity rates were significantly decreased by the low radiation dose and increased administering times. The results suggest that surgery in conjunction with an adjunct low-dose radiation treatment was effective for creating a reliable and sustained lymphedema model that could be applied for further study.

Creating a sustained lymphedema model has always been challenging. Previous studies demonstrated the incapability of surgery to create a more sustained model. Problems arose from the vigorous regeneration of lymphatic endothelial cells (LECs) that resulted in spontaneous lymphatic reconnection. According to Joseph et al, inflammatory response following lymphadenectomy surgery could promote lymphangiogenesis through the release of lymphangiogenic cytokines and activation of stem cells [22]. The result implied that lymphedema was barely sustained without additional intervention. To solve this problem, radiation treatment was introduced. Lee-Donaldson et al [16] and Park et al [13] prolonged lymphedema in animal models by administering the radiation treatment either before or after the surgical treatment. However, they reported a high mortality rate and serious limb necrosis, which may be caused by suboptimal radiation dose. A modified way of administering radiation to induce stable lymphedema without serious adverse effect is urgently needed. Therefore, in our study, three strategies were designed to determine the optimal combination of radiation and surgery as follows: 4.5-Gy radiation treatment administered 3 days before surgery, 4.5-Gy radiation treatment administered 2 weeks after surgery, and two doses of 2.25-Gy radiation treatment administered 3 days before surgery and 2 weeks after surgery, respectively. The results showed lymphedema formation in all the three groups, with the r+S+r group showing the most obvious and longest enduring edemas. In addition, the r+S+r group showed the lowest mortality rate, without limb necrosis. In summary, this study demonstrates that the

optimal radiation condition could be reached through adjustment of the timing of the administration of radiation (3 days before and 2 weeks after surgery) and by reducing the dose (2.25 Gy).

A previous study reported that radiation contributed to the formation of lymphedema owing to its damage to LECs, which increased permeability and apoptosis, and the overproduction of TGF-beta, which inhibited LEC proliferation [23]. Therefore, radiation treatment before surgery may damage or inhibit the growth of existing LECs and stem cells, which would be involved in lymphangiogenesis after surgical stimulation. However, the effect of promoting lymphedema formation could be counteracted by activation of lymphangiogenesis after surgery so that the inhibition was largely weakened. This could be the reason that the R+S group showed the highest volume increase in the first week after surgery, but the edematous state subsided later, so that a stable lymphedema model could not be formed. When administered after surgery, radiation treatment suppressed postoperative activated lymphangiogenesis and release of cytokines such as lymphangiogenic growth factor vascular endothelial growth factor C, which could increase radiosensitivity of LECs, as previously reported [24]. This was reflected in the intensification of the lymphedema in the S+R group, which was sustained for longer time after postoperative radiation treatment. Unfortunately, the toxicity of the 4.5-Gy radiation dose together with surgical injury may lead to high rates of animal mortality and limb necrosis. Therefore, we evenly divided the total dose of 4.5 Gy into 2 dosing times as follows: 3 days before surgery and 2 weeks after surgery. In effect, the suppression of lymphangiogenesis was augmented and limb necrosis caused by a single high dose of radiation was prevented. In addition, as repeated radiation could increase the cells' radiosensitivity [25], the inhibiting effect was greatly enhanced. Therefore, a high volumetric peak was reached immediately in the acute lymphedema phase after surgery. However, later, instead of quickly subsiding, the edematous state persisted with a relatively high volume for 24 weeks and low adverse events such as animal death and limb necrosis. Lymphatic mapping in vivo and the difference in lymphatic vessel density in vitro further confirmed the weaker lymphangiogenesis in the r+S+r groups, which

accounted for the formation of sustained lymphedema. Moreover, the specific timing for radiation treatment played an important role in the formation of stable lymphedema. Radiation treatment was administered 3 days before surgery mainly because the destructive effect of radiation exposure on lymphatic tissue begins 3 days after radiation [26], which could reinforce the radial obstruction of lymphatics. The time point for the second radiation was at 2 weeks after surgery because we found that postoperative activated lymphangiogenesis mostly occurred at this time point, which was also demonstrated by previous studies [27]. Although the total dose of 4.5 Gy was also used in previous studies, toxicity was substantially reduced by administering the dose at two time points. Thus, we believe that the above-mentioned strategy may serve as an optimal method for lymphedema animal models.

Conclusion

We, for the first time, adopted a “low-dose radiation + surgery + lower-dose radiation” strategy for successfully establishing mouse lymphedema models. A more important finding is that the lymphedema model was sustained longer than other previously reported models, with low mortality and morbidity rates. We believe the model met the requirement for further lymphedema study.

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Disclosure of conflict of interest

None.

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