Master of Medicine

Evaluation of the effects of pre-injury exercise in a mouse hindlimb model of secondary lymphedema

The Graduate School

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Department of Medicine

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Evaluation of the effects of pre-injury exercise in a mouse hindlimb model of secondary lymphedema

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This certifies that the dissertation of Ma. Nessa Gelvosa is approved.

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-Ma. Nessa Gelvosa

Abstract

Background and Purpose. Lymphedema is a common but distressing sequela after cancer treatment. A curative treatment is not yet available; thus, prevention is a vital part of its management. This study aims to determine the effect of exercise before injury, i.e., lymph node resection and radiation, on the development of lymphedema in mice hindlimb.

Methods. BALB/c mice were randomized into 2 groups: exercise (n=7) and no exercise (n=7). The exercise group performed 30 minutes of moderate exercise on a treadmill, 5 days a week for 4 weeks, while the no exercise group was placed on a stationary treadmill for the same amount of time. Lymphedema was induced by unilateral popliteal lymph node resection and 20 Gy lower limb irradiation. Caliper measurements of ankle diameter and hindpaw thickness were obtained weekly for 6 weeks, and the percentages of excess ankle diameter (PED) and hindpaw thickness (PET) relative to the intact control limb were calculated. Weekly indocyanine green (ICG) lymphangio-imaging was performed to visualize dermal backflow patterns. Histopathological analysis on the 6^{th} week post injury was also done to evaluate skin thickness and lymphatic vessel density.

Results. The exercise group exhibited a significantly lower PED compared to the no exercise group at weeks 3, 4, 5, and 6 after injury, as well as significantly lower PET at weeks 5 and 6 after injury. ICG imaging showed that the no exercise group had a significantly higher proportion of mice demonstrating the more severe stardust and diffuse patterns at weeks 1, 4 and 5 after injury. On hematoxylin and eosin slides, the exercise group showed a significantly lower increase in epidermal and dermal thickness. On immunohistochemistry staining with anti-LYVE-1, the exercise group showed a higher lymphatic vessel density than the no exercise group.

Conclusion. Exercise before lymphatic injury may attenuate the development of secondary lymphedema induced by lymph node resection and radiation in mice hindlimb. This may have implications for the role of prehabilitation in human lymphedema.

Keywords: physical activity, prehabilitation, lymphangiogenesis, secondary lymphedema

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Introduction

Secondary lymphedema is one of the most common and devastating sequelae of oncologic surgery and radiation. Damage to the lymphatic system brought about by these procedures lead to accumulation of protein-rich fluid in the interstitial space causing immense volume increase in the affected limb, abnormal fat deposition, fibrosis, chronic inflammation, marked skin pathology and recurrent infections [1]. Symptoms of lymphedema include pain or discomfort, limb heaviness, weakness, restricted range of motion, obvious deformity and psychologic morbidities including anxiety, depression, and negative body image, which ultimately decrease patients' quality of life [2].

Lymphedema continues to affect 1 in 7 patients treated for cancer despite recent advances in cancer treatment [3]. The incidence of lymphedema in breast cancer, which is the most common cause of upper extremity lymphedema, approaches 20% following axillary lymph node clearance [4]. In the case of lower extremity lymphedema, gynecologic cancers are a common cause in women and incidence is 37% by 24 months post-gynecologic cancer surgery [5].

Despite being a common condition with a significant burden of disease, there is still an unmet need for a curative treatment. The current standard of care for lymphedema is complete decongestive therapy, which combines manual lymphatic drainage, compression strategies, remedial exercises and skin care. However, this also puts restrictions on the patient's quality of life as it is time-consuming, expensive, and requires lifelong maintenance. Considering this, as well as the chronic and progressive nature of lymphedema, prevention or prehabilitation is an important consideration in the management of lymphedema.

Prehabilitation refers to multimodal interventions applied in anticipation of an upcoming stressor, such as surgery or cancer treatment, in order to reduce the associated side effects, functional decline and impairments [6]. Exercise is a key component of prehabilitation and rehabilitation in general. Historically, patients with lymphedema were discouraged to exercise in fear of aggravating the swelling due to increased production of interstitial fluid during exercise [7]. However, this outdated belief has already been challenged by numerous clinical studies which has established the safety of exercise in lymphedema and has shown that exercise does not affect limb swelling, but can even reduce

lymphedema symptoms and exacerbations [8-11]. More studies are still needed to fully elucidate the role of exercise in lymphedema, including determining the specific exercise parameters that would be most beneficial in this condition. Insights on structural and functional changes in response to exercise and possible mechanisms need further investigation. For these applications, animal studies remain useful.

With the safety of exercise already established, recent studies shift focus on the possible therapeutic or preventive role of exercise in lymphedema [7]. Most studies on exercise as a preventive strategy, however, applied exercise after lymphatic injury, that is after surgery or radiation but before swelling and lymphedema appeared. There is limited to nil information about the effect of exercise performed before lymphatic injury. This period may be crucial as it has been shown that baseline lymphatic dysfunction amplifies the negative effects of lymphatic injury [12], therefore risk reduction strategies and therapeutic measures to increase lymphatic functional reserve prior to surgery or radiation, such as exercise, should be considered during prehabilitation, especially for patients with known risk factors and identified lymphatic dysfunction.

This study aimed to evaluate the effects of pre-injury exercise in a mouse hindlimb of lymphedema. Specifically, it aimed to compare the development of lymphedema induced by surgery and radiation in mice which performed pre-injury exercise and mice which did not exercise, in terms of severity of swelling, indocyanine green lymphangio-imaging patterns and histopathologic findings such as skin thickness and lymph vessel density.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of our institution and conformed to the U.S. National Institutes of Health Guidelines on the care and use of laboratory animals. The overall flow of the study is depicted in figure 1.

Animals

A total of 14 male BALB/c mice (Orient Bio, Seongnam, Korea), aged 8-12 weeks, and weighing approximately 23-27g were used. Mice were maintained in a light- and temperature-controlled environment and given free access to water and laboratory chow *ad libitum*.

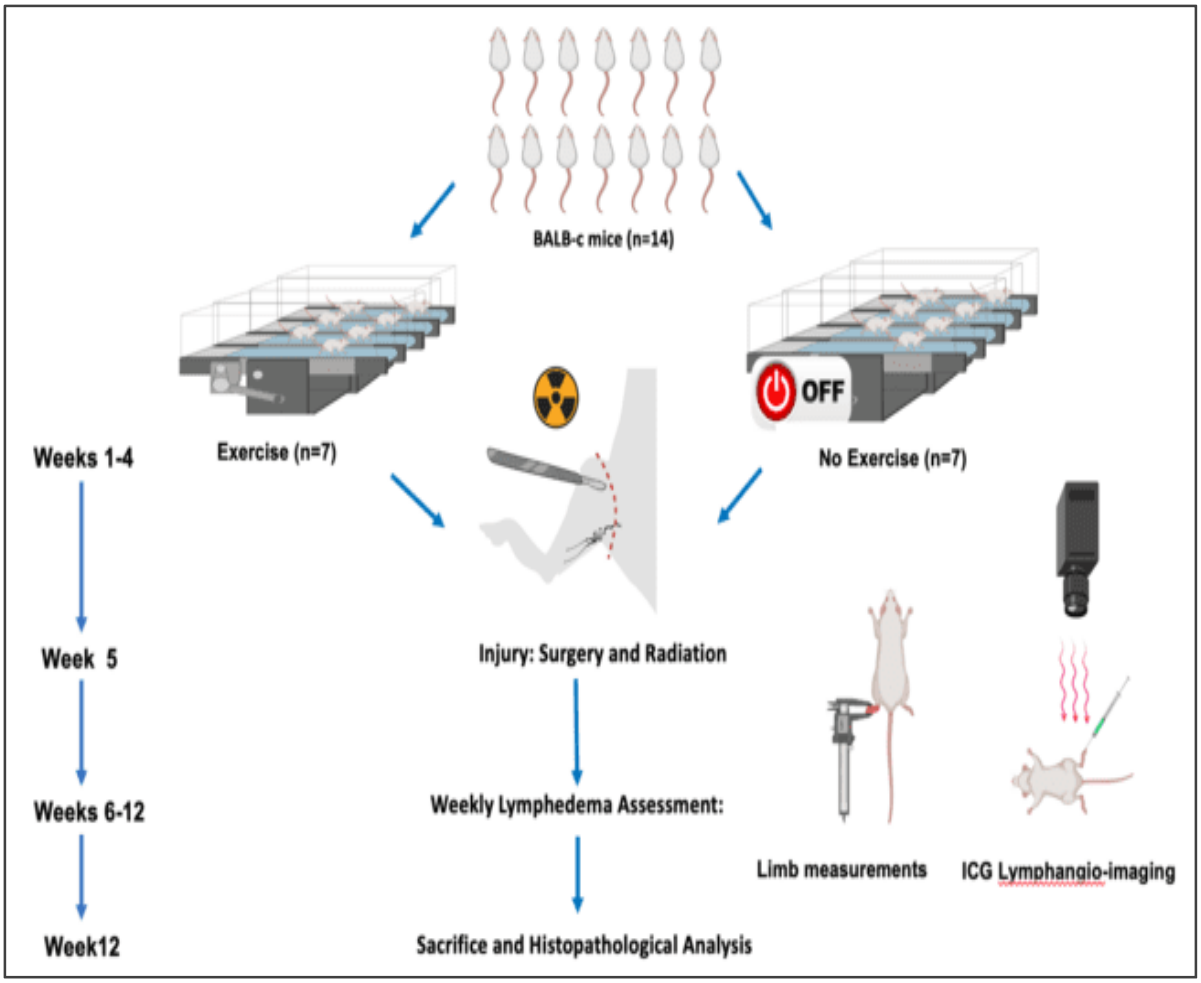

Figure 1. Flow of Study

Exercise Protocol

The mice were randomly allocated into 2 groups: exercise (n=7) and no exercise (n=7) groups. The exercise group performed treadmill exercise (fig. 2) for 4 weeks using a modified protocol previously described by [13]. One week prior to the exercise treatment proper, the exercise group was acclimatized to the treadmill (Rodent treadmill, Howasung,Seoul, Korea) for 10 minutes a day. They were started at 0m/min and speed was gradually increased every 2 minutes until 10m/min for the last 2 minutes of acclimatization. For the exercise treatment proper, the exercise group ran on the treadmill at a moderate intensity for 30 minutes a day, 5 days a week, for 4 weeks. Mice were stimulated by lightly tapping on the treadmill cover to produce sound to ensure that they run continuously. The no exercise group was subjected to similar handling but was placed on a sedentary treadmill for the same amount of time. Moderate intensity is defined as 45-55% of maximum speed from incremental load test performed prior to the start of the exercise training period [14]. The maximum speed was determined by increasing the intensity of the exercise by 3min/min every 3 minutes at 0% grade until exhaustion [14, 15].

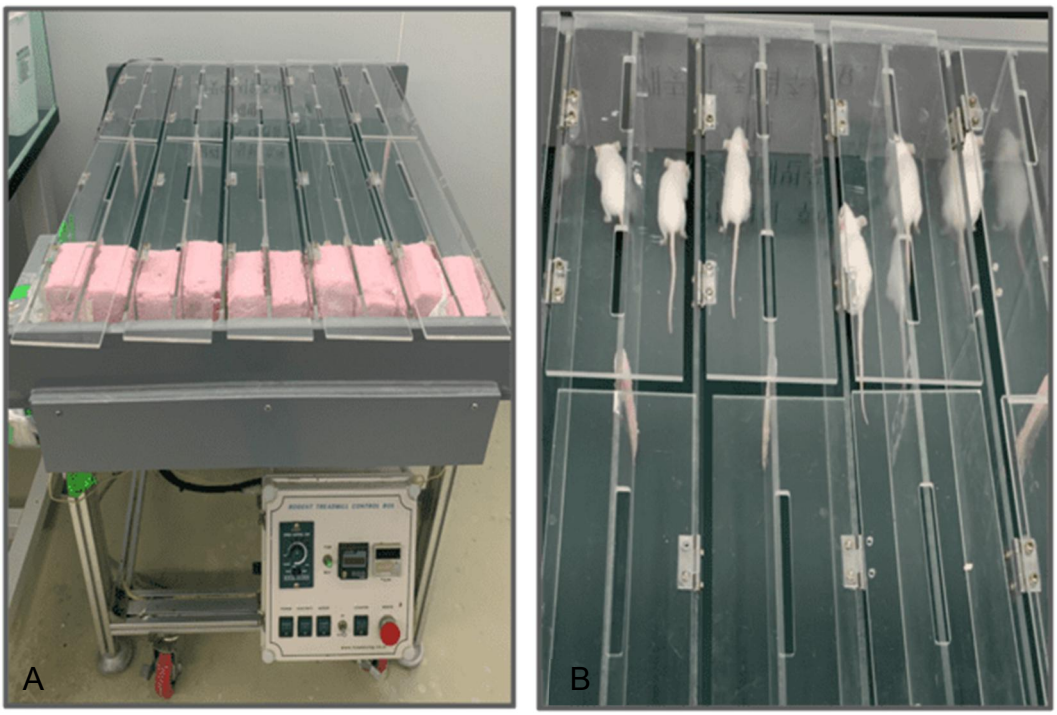

Figure 2. A. shows the motorized rodent treadmill used in this study. B shows mice exercising on the treadmill lanes.

Lymphedema Induction

After the 4-week course of exercise, unilateral hindlimb lymphedema (fig. 3) was induced using a modification of a previously described protocol [16]. One investigator perfomed all surgical procedures. Mice were anesthetized through intraperitoneal injection of 50 mg/kg zolazepam and tiletamine (Zoletil 50; Virbac, Carros, France) and 10 mg/kg xylazine (Rompun; Bayer HealthCare, Leverkusen, Germany). Depth of anesthesia was monitored regularly through toe pinch and observation of respiratory rate and pattern. Once mice were adequately anesthetized, shaving was done and then residual hair was removed using depilatory cream (Tosowoong hair removal cream, South Korea). A 10-uL volume of 1% Evans blue (Sigma-Aldrich, St. Louis, MO, USA) was injected subcutaneously between the 2nd and 3rd toes to locate the popliteal lymph node. The skin near the popliteal fossa was then circumferentially incised, and the popliteal lymph node was resected along with the surrounding adipose tissue. The afferent and efferent lymph vessels were obliterated using Bovie cautery. Wound edges were then folded and sutured using 4-0 nylon (fig. 4). Two days after the surgery, mice were again anesthetized, and the operated limb was subjected to a single dose of 20 Gy of radiation from an X-Rad 320 machine (PXi, North Branford, CT, USA) (fig. 5). The contralateral limb was left intact to serve as control.

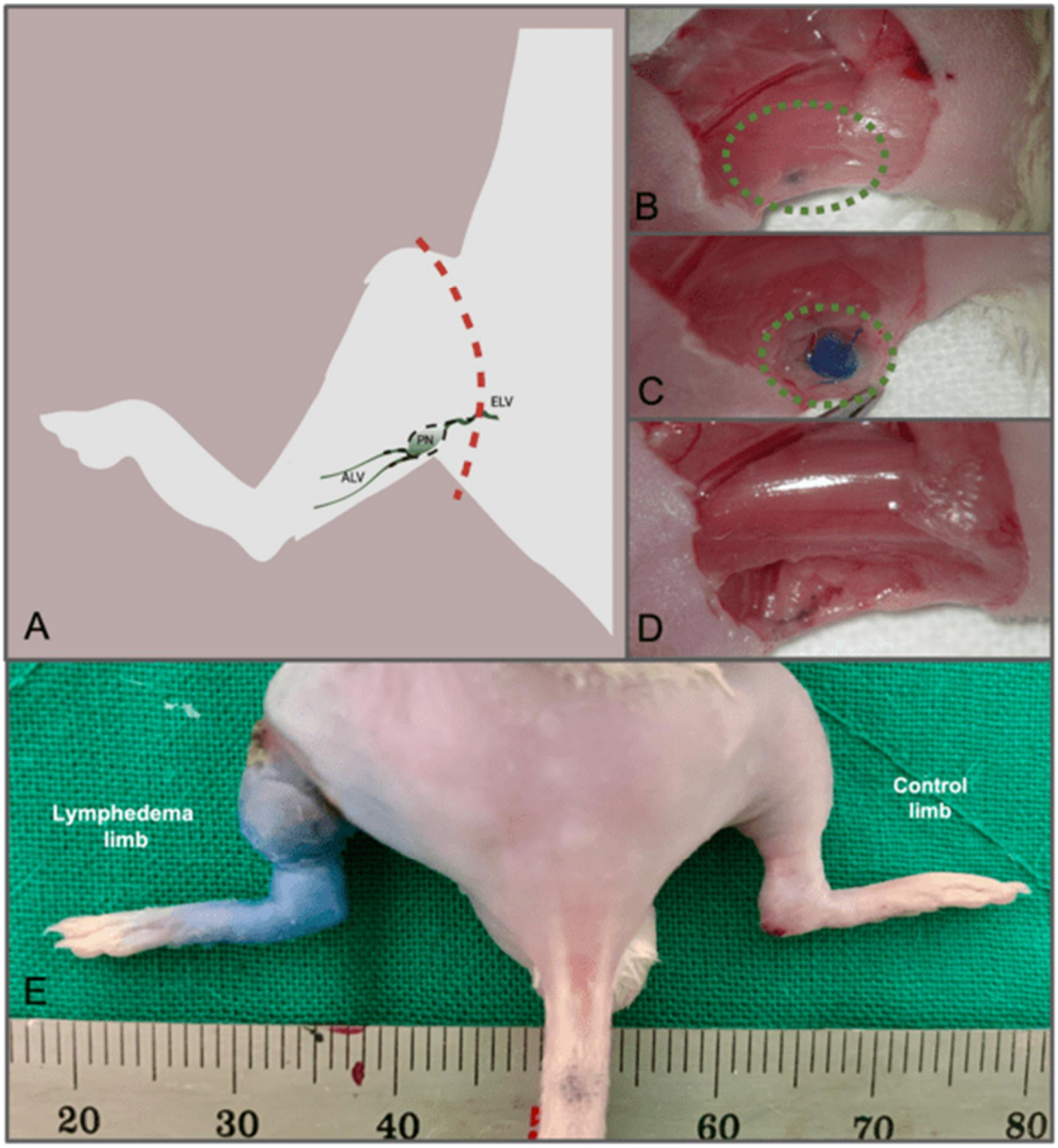

Figure 3. Mouse hindlimb model of lymphedema. A. Schematic diagram of the mouse hindlimb showing the two afferent lymphatic vessels (ALV) leading to the popliteal lymph node (PN) and one efferent lymphatic vessel (ELV). The red dashed line indicates the site of circumferential skin incision. B. The green dashed circle surrounds the popliteal lymph node which is covered by fascia and adipose tissue. C. The popliteal lymph node appears blue as stained with Evans blue dye. D. Resection of the popliteal lymph node and surrounding fat leaves a gap in the tissue. E. A representative image of a hindlimb model of lymphedema one week after surgery and radiation.

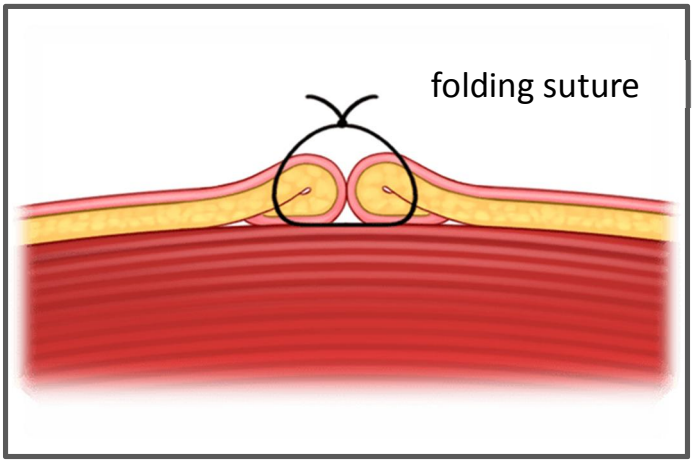

Figure 4. The skin edges were folded before they were closed by interrupted sutures in an attempt to prevent reconnection of dermal lymphatic vessels.

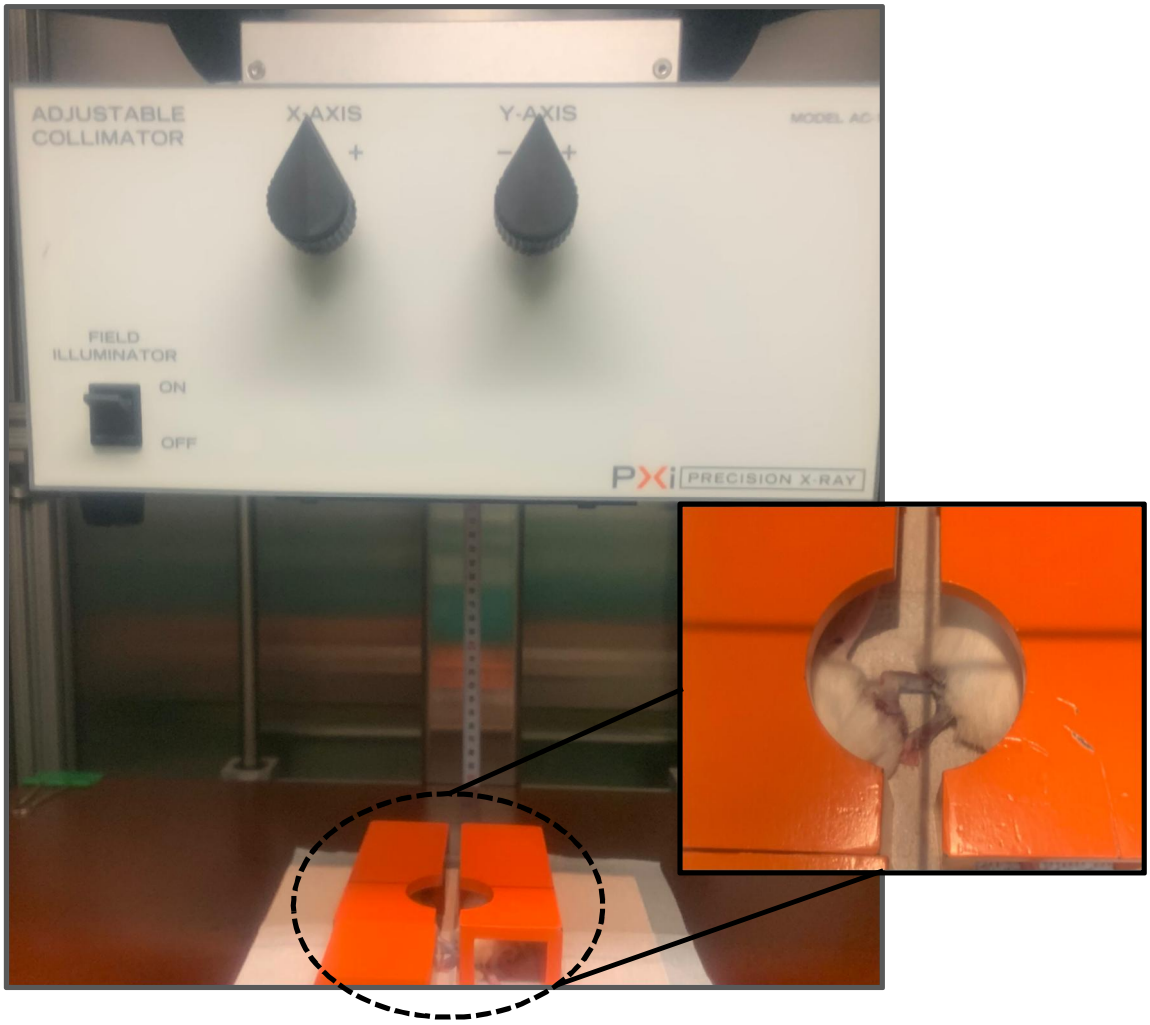

Figure 5. The operated limbs were exposed to a single dose of 20 Gy of radiation 2 days after the surgery. The unaffected parts of the mice were covered with lead blocks.

Lymphedema Assessment

Measurements were done by one investigator blinded to the treatment groups. Ankle diameter and hindpaw thickness were measured using a digital caliper at standardized locations at all time points. Ankle diameter was measured at 5mm proximal to heel, while hindpaw thickness was taken between the first and second proximal pad of the paw. Percentage of excess ankle diameter (PED) and percentage of excess hindpaw thickness (PET) of the lymphedematous limb relative to the control limb were then calculated to express the severity of swelling and to account for age-appropriate growth and differences in animal size. PED and PET were modifications of percentage of excess volume used in previous studies [17-20] and were computed as follows:

$$\text{PED} = \frac{\text{ankle diameter}_{\text{injury limb}} - \text{ankle diameter}_{\text{control limb}}}{\text{ankle diameter}_{\text{control limb}}} \times 100\%$$

$$\text{PET} = \frac{\text{hindpaw thickness}_{\text{injury limb}} - \text{hindpaw thickness}_{\text{control limb}}}{\text{hindpaw thickness}_{\text{control limb}}} \times 100\%$$

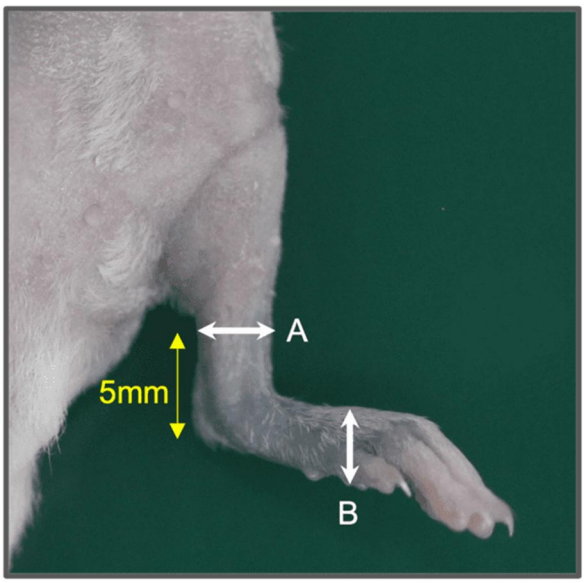

Figure 6. Standardized location of measurement of ankle diameter (A) and hindpaw (thickness)

Indocyanine Green Lymphangio-imaging

We performed indocyanine green lymphangio-imaging (ICG) every week to visualize and compare the lymphatic patterns between both groups over a period of 6 weeks. Mice were anesthetized using the procedure described earlier and hair was removed prior to each imaging session. Two microliters of indocyanine green (Sigma-Aldrich, St. Louis, Missouri, USA), dissolved in distilled water to make a 1mg/ml solution, was injected intradermally into the dorsal aspect of both hindpaws using a 31-gauge needle. Images were taken 15 minutes after ICG injection using a near-infrared fluorescence camera system (Moment K, IANC&S, Seoul, Korea). Since there were several patterns visualized on one limb, the ICG imaging pattern on the dorsal proximal area, near the area of the resected lymph node was chosen and classified according to increasing severity of lymphedema: linear (normal), splash (mild), stardust (moderate) and diffuse (severe), based on a previous study [21].

Histopathology and Immunohistochemistry

At 6 weeks post-injury, mice were sacrificed by carbon dioxide asphyxiation using the protocol recommended in the Guidelines on Euthanasia by the American Veterinary Medical Association. Both hindlimbs were harvested and fixed in 10% neutral buffered formalin at 4°C for 24 hours. Specimens were then decalcified in decalcifying solution (Calci-Clear Rapid Histological Decalcifying Reagent, National Diagnostics, GA, USA) for at least 48 hours. After decalcification, tissue samples were obtained at approximately 6 mm proximal to heel, and at 10mm distal to heel, then embedded into paraffin blocks using a paraffin embedding station (Leica EG1150H, Leica Biosystems, IL, USA). The paraffin blocks were then cut into 3-µm-thick sections on a rotary microtome (Leica RM2255, Leica Biosystems, IL, USA). Sections were prepared onto glass slides. Hematoxylin and eosin (H&E) staining and mounting with coverslips were performed using an automatic stainer (Leica autostainer XL, Leica Biosystems, IL, USA). Immunohistochemisty staining of lymphatic vessels was done using anti-lymphatic vessel endothelial hyaluronan receptor receptor-1 (anti-LYVE-1) antibody at a dilution of 1:100 (ab14917, Abcam, Cambridge, United Kingdom).

Skin thickness was measured from the H&E slides. Tissue sections were observed using the 20X objective of an Olympus BX53 microscope with an integrated DP-27 digital camera (Olympus, Tokyo, Japan). Epidermal and dermal thickness were measured at a minimum of 3 random high power fields in the dorsal hindpaw, in the area bounded laterally by the 3 middle metatarsals.

For the IHC evaluation, stained lymphatic vessels between the epidermis and muscle layers were counted at a minimum of 3 random high-power fields in the dorsal hindpaw in the region of interest used in the skin thickness evaluation. For the ankle, the entire field was divided into 4 quadrants and the number of lymphatic vessels were counted at 3 random high power field per quadrant. The mean number of lymphatic vessels in the 4 quadrants was recorded for each specimen.

All images were processed and evaluated using a digital microscope program (Olympus Cellsens Standard ver. 1.13, Olympus, Tokyo, Japan) in a blinded manner.

Statistical Analysis

Data were presented as mean ± standard deviation, unless otherwise indicated. Statistical analysis was performed using Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). Due to the small number of samples, Mann-Whitney test, a non-parametric test, was used to compare the differences in means between the two groups at a single time point. Fisher's exact test computed by the VassarStats Website for Statistical Computation was used to compare the difference in proportions between the two groups. A p-value < 0.05 was considered significant.

Results

Lymphedema Assessment

All mice developed lymphedema immediately after surgery and radiation (fig. 7). Percentage in excess of ankle diameter (PED) peaked on the 1st week after injury for both groups and decreased thereafter until the 3rd week when it started to plateau until the 6th week (fig. 8). PED of the exercise group was consistently lower compared to the no exercise group throughout the entire follow-up period, and the differences were noted to be significant at week 3 (7.48% ± 4.35 vs 14.65% ± 5.69, p-value=0.017), week 4 (5.88% ± 2.80 vs 11.35% ± 3.34, p-value=0.026), week 5 (4.51% ± 2.49 vs 14.65% ± 5.62, p-value=0.002) and week 6 (4.30% ± 2.17 vs 12.55% ± 4.63, p-value=0.007) (table 1). Moreover, the percentage in excess of hindpaw thickness (PET) was lower for the exercise group compared to the no exercise group at weeks 3-6 (fig. 9), but the differences were significant only in week 5 (12.08%± 4.22 vs 22.28%, p-value=0.041) and week 6 (10.55% ± 3.53 vs 17.20% ± 5.01, p-value=0.011) (table 1).

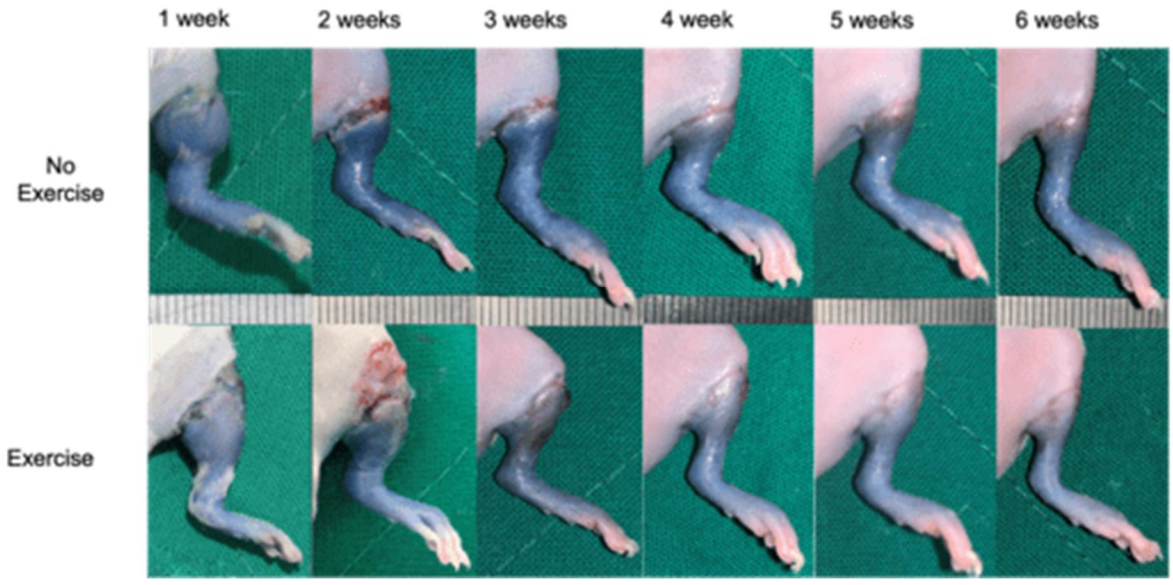

Figure 7. Gross pictures of the operated limbs of the no exercise (top) and exercise (bottom) groups over the 6-week observation period.

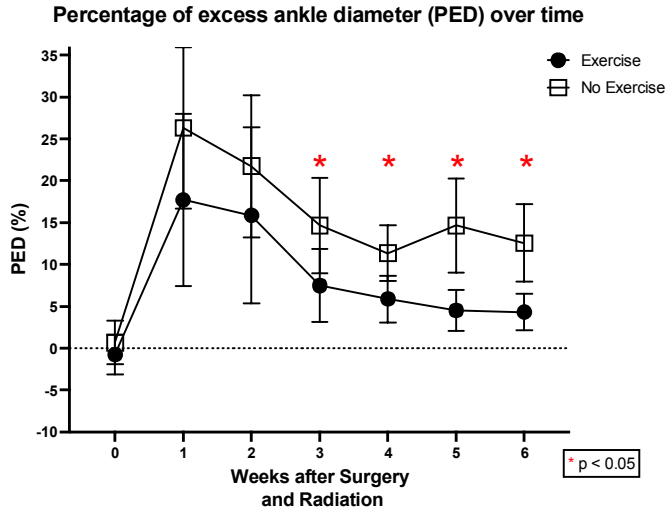

Figure 8. Percentage of excess ankle diameter (PED) of the exercise and no exercise groups over the 6-week follow-up period. (n=7/group)

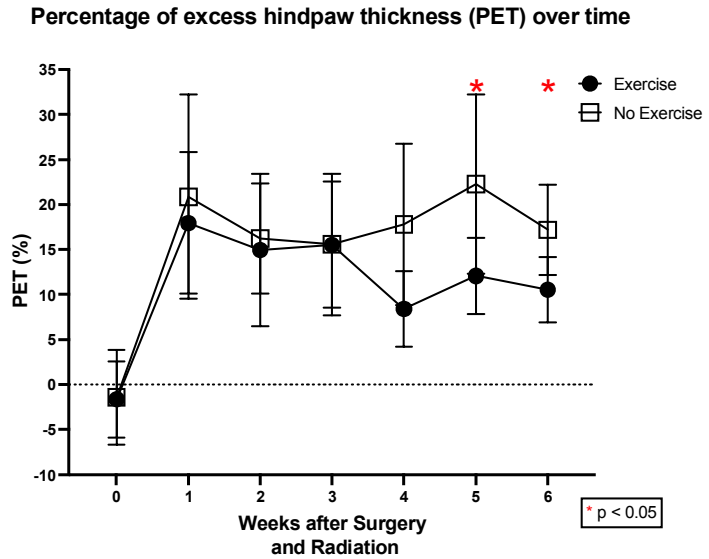

Figure 9. Percentage of excess hindpaw thickness (PET) of the exercise and no exercise groups over the 6-week follow-up period. (n=7/group)

Table 1. Percentage of excess ankle diameter (PED) and percentage of excess hindpaw thickness (PET) of the exercise and no exercise groups over the 6-week follow-up period

Weeks after injury	Percentage of excess ankle diameter (PED)			Percentage of excess hindpaw thickness (PET)		
	Exercise (n=7)	*No Exercise (n=7)*	*p-value* †	*Exercise (n=7)*	*No Exercise (n=7)*	*p-value* †
0	-0.75 ± 2.36	0.66 ± 2.59	0.335	-1.65 ± 4.24	-1.39 ± 5.29	0.901
1	17.71 ± 10.29	26.28 ± 9.63	0.097	17.96 ± 7.85	20.86 ± 11.36	0.901
2	15.87 ± 10.53	21.74 ± 8.49	0.209	14.96 ± 8.44	16.24 ± 6.12	0.710
3	7.48 ± 4.35	14.65 ± 5.69	0.017*	15.55 ± 7.01	15.57 ± 7.87	>0.999
4	5.88 ± 2.80	11.35 ± 3.34	0.026*	8.41 ± 4.20	17.81 ± 8.98	0.073
5	4.51 ± 2.49	14.65 ± 5.62	0.002*	12.08 ± 4.22	22.28 ± 9.99	0.041*
6	4.30 ± 2.17	12.55 ± 4.63	0.007*	10.55 ± 3.53	17.20 ± 5.01	0.011*

† *Mann-Whitney test*

**p-value < 0.05*

Indocyanine Green Lymphangio-imaging

There was a significant difference noted in dermal backflow patterns demonstrated by the exercise and no exercise at weeks 1, 4 and 5 post-injury with more mice in the no exercise group exhibiting splash and diffuse patterns which are considered more severe patterns (p-values at 0.013, 0.038, 0.008, respectively) (fig. 10,11). At the first week post-injury, 5 (71%) of the no exercise mice showed diffuse patterns and 2 (29%) showed stardust patterns, while 3 (43%) of the exercise mice showed splash patterns and 4 (57%) showed stardust patterns. At week 3, four (57%) of the no exercise mice still had a diffuse pattern and 2 (29%) showed stardust pattern, while the exercise group started demonstrating a linear pattern (14%) and none had a diffuse pattern. At week 5, majority of the exercise mice (86%) exhibited the splash pattern, while the no exercise group still had the diffuse (43%) and stardust (29%) patterns. At the end of the 6th week observation period, although the difference was not statistically significant, the no exercise group still had mice with diffuse (29%) and stardust (29%) patterns while the exercise group only showed linear (14%) and splash (71%) patterns.

Aside from dermal backflow patterns, collateral flow to the inguinal lymph node (fig. 12) was also observed in a greater proportion of exercise mice at weeks 3, 5 and 6 post-injury (table 2), although the differences were not statistically significant.

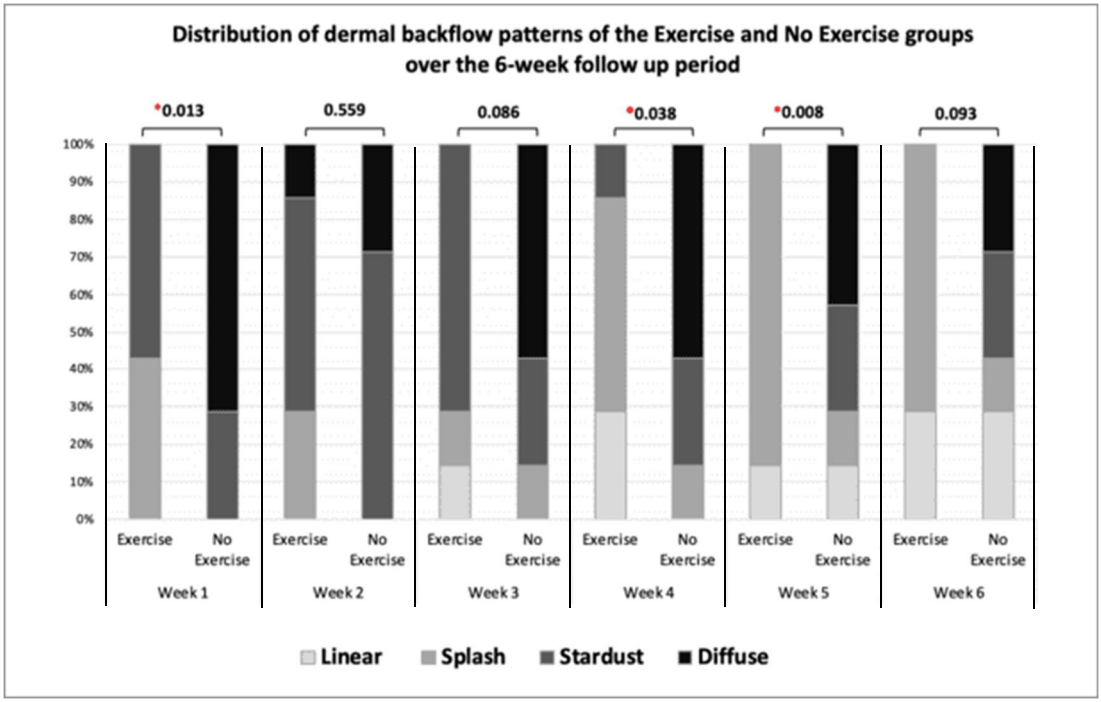

Figure 11 . Proportion of mice demonstrating each dermal backflow pattern at each follow-up period (n=7/group)

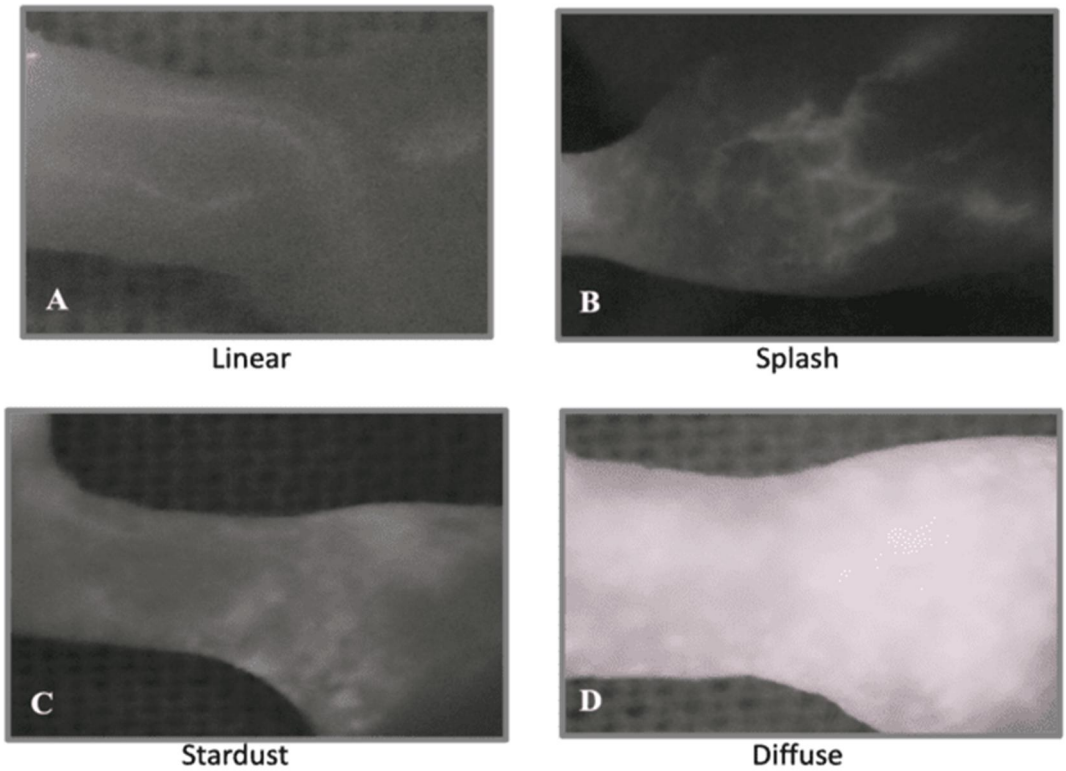

Figure 10. Representative images of the dermal backflow patterns visualized on indocyanine green lymphangio-imaging of mouse hindlimb lymphedema model. These are arranged according to severity of lymphedema: A: linear (normal), B: splash (mild), C: stardust (moderate), D: diffuse (severe)

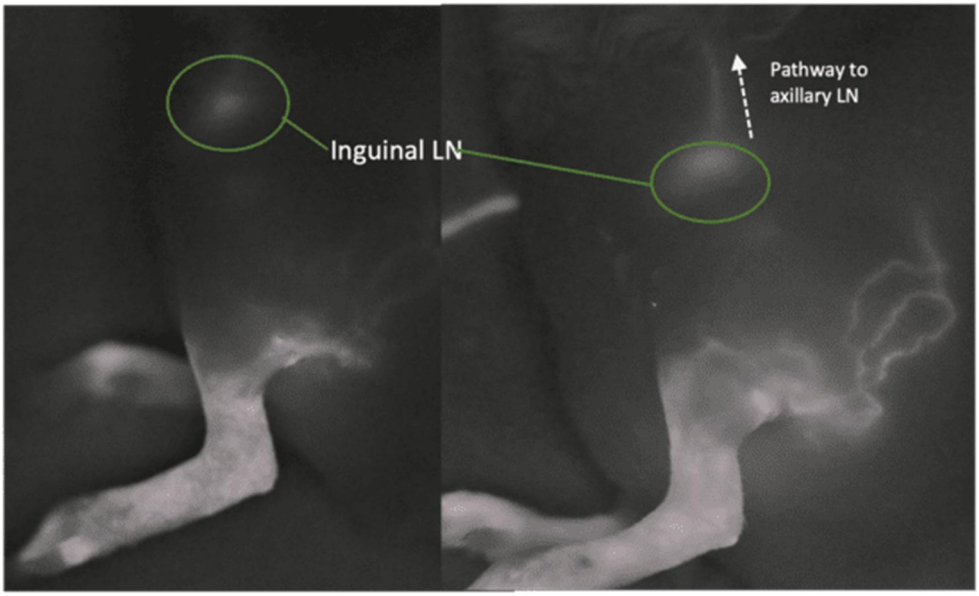

Figure 12. Collateral flow from the hindlimb to the inguinal lymph node and subsequently towards the axillary lymph node in a mouse from exercise group. The pathway to the inguinal lymph node is a novel pathway in mice which occurs after popliteal LN resection. Lymph may also flow to the axillary LN from the inguinal LN, but this pathway is inefficient in mice.

Table 2. Proportion of mice in each group with collateral lymph vessels draining to the inguinal lymph node over the 6-week observational period after surgery and radiation.

Weeks after injury	**Exercise (n=7)**	**No Exercise (n=7)**	**p-value**
1	0/7	0/7	1
2	0/7	0/7	1
3	3/7	2/7	0.999
4	3/7	3/7	1
5	5/7	3/7	0.592
6	5/7	4/7	0.999

Histopathology and Immunohistochemistry

As shown in figure 13, the exercise group showed a significantly lower increase in both epidermal thickness (6.81μm ± 3.22 vs 13.21 μm ± 3.79, p-value=0.0152) and dermal thickness (126.39 μm ± 47.41 vs 289.39 μm ± 112. 84, p-value-=0.0152) compared to the no exercise group. Representative H&E images are shown in figure 14. Aside from hyperkeratosis and dermal expansion, the no exercise hindpaw skin also showed dilated lymphatic vessels (fig 14D).

For the immunohistochemistry evaluation of the lymphatic vessels in the dermis/subcutaneous tissue (figs. 15 and 16) of the injury limbs, the exercise group exhibited a greater number of lymphatic vessels per high power field in the hindpaw (10.01 ± 2.26 vs 7.34 ± 2.41, p=value 0.051) and ankle (9.81 ± 2.76 vs 6.64 ± 1.64, p-value=0.035), but the difference was only statistically significant in the latter.

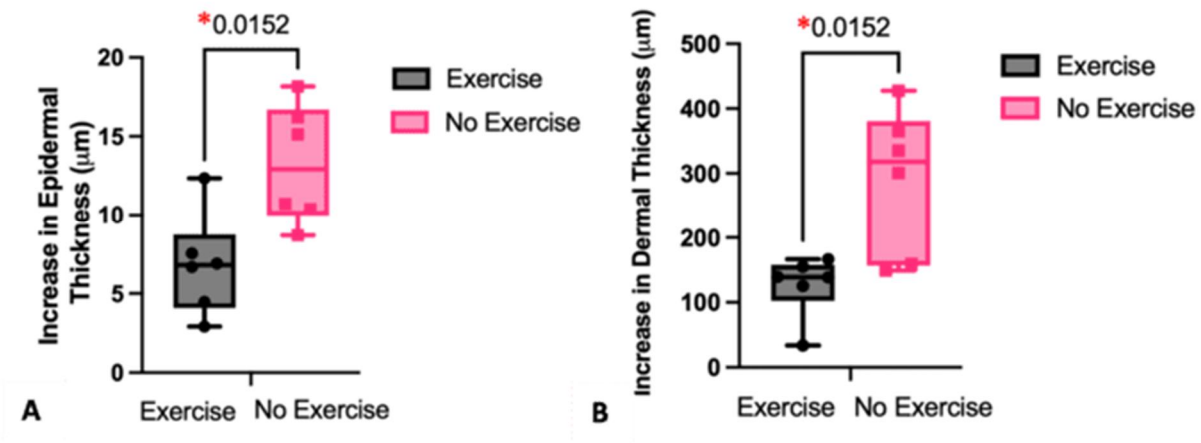

Figure 13. Mean increase in epidermal (A) and dermal (B) thickness in the hindpaw of the injury limb relative to the control limb of the exercise and no exercise groups at 6 weeks after surgery and radiation (n=6/group)

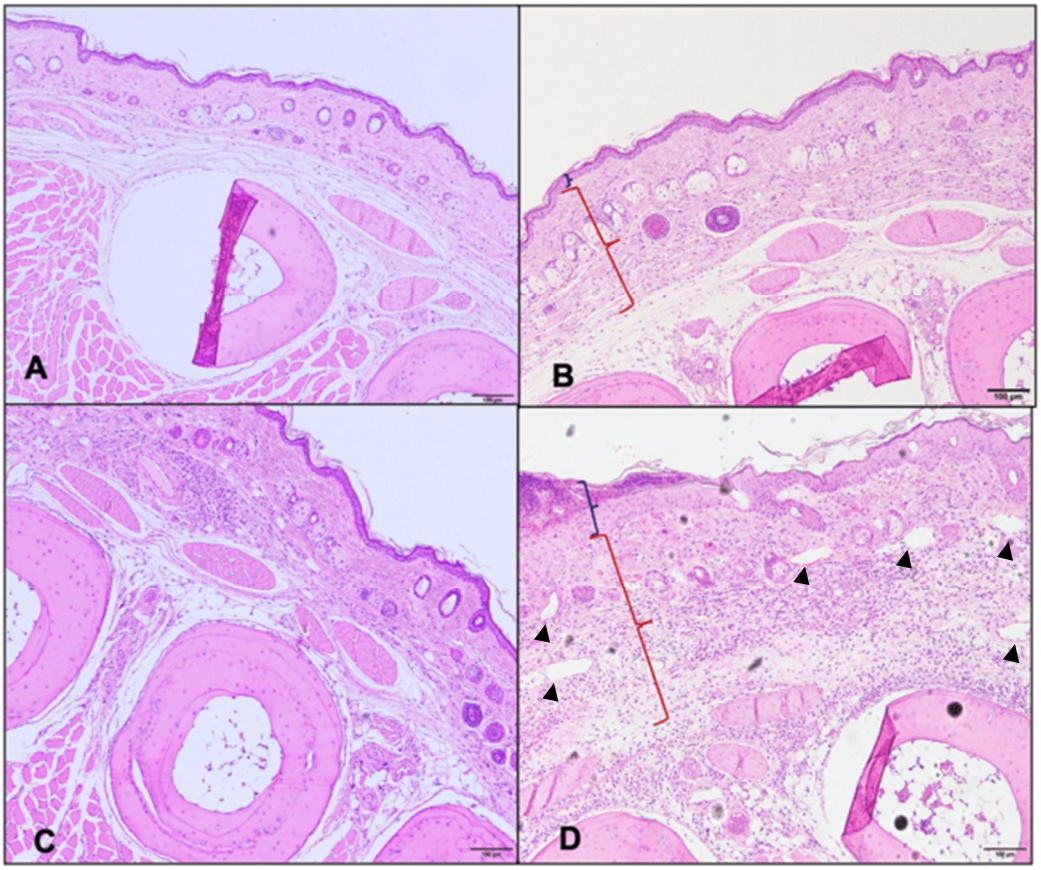

Figure 14. Representative H&E images of control limbs of exercise (A) and no exercise (C) mice, and injury limbs of exercise (B) and no exercise (D) mice. Dilated lymphatic vessels (arrowheads) are seen in D. Blue brackets indicate epidermal thickness while red brackets indicate dermal thickness.

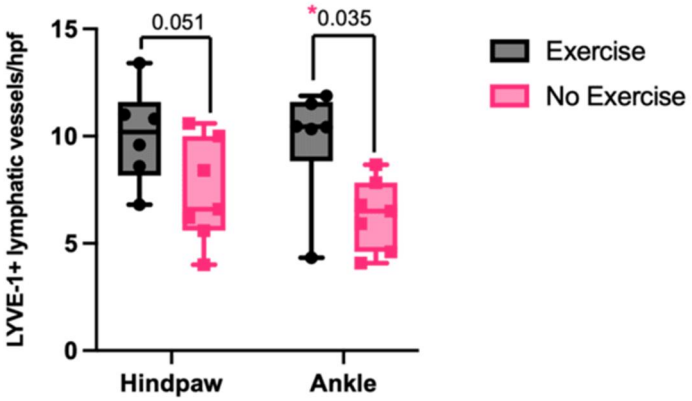

Figure 15. Average number of lymphatic vessels per high power field in the dermis/subcutaneous tissue of injury limb of both exercise and no exercise groups at 6 weeks after surgery and radiation (n=6/group). LYVE-1 (Lymphatic vessel endothelial cell-1)

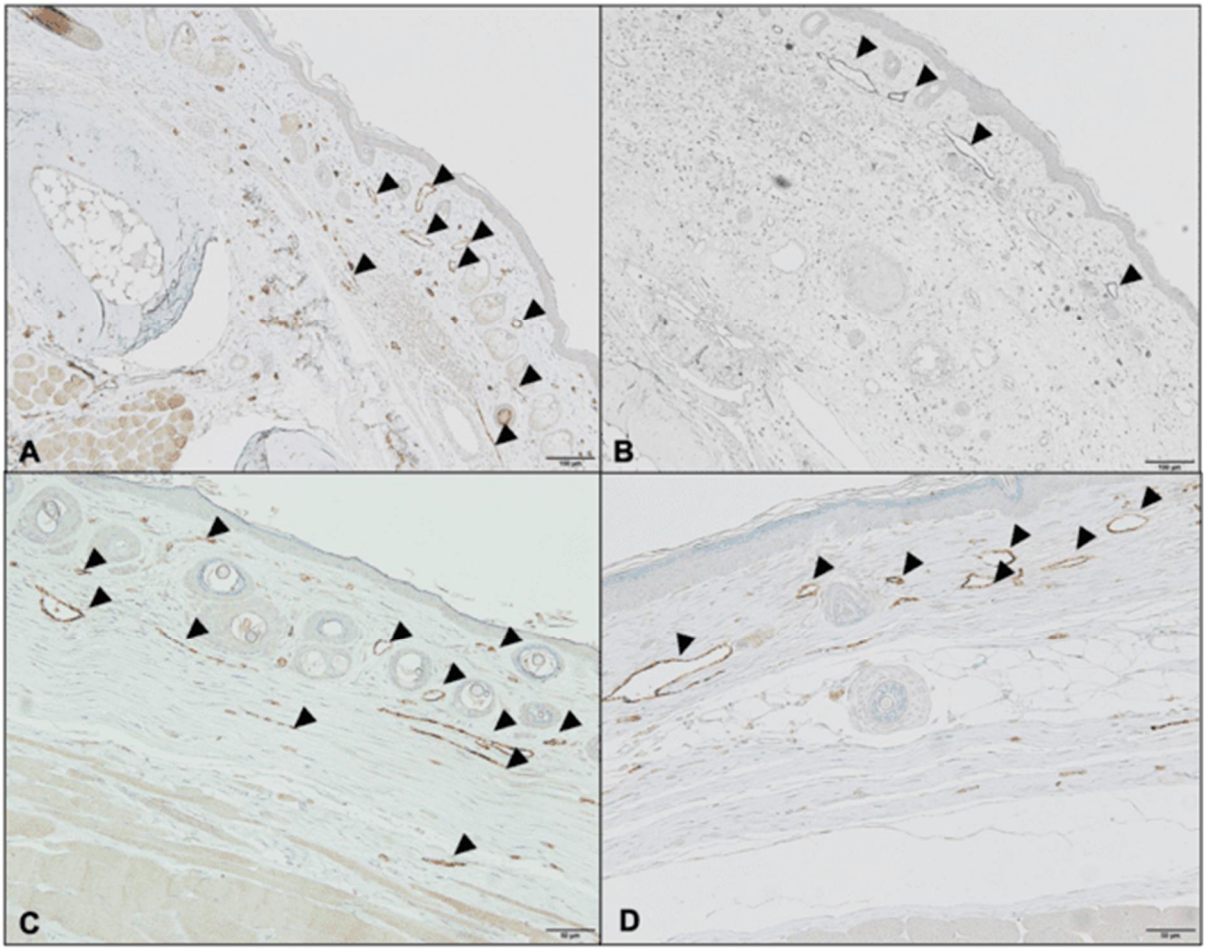

Figure 16. Representative immunohistochemistry (anti-LYVE-1) slides showing lymphatic vessels (arrow heads) in the hindpaw of exercise (A) and no exercise (B) mice, and ankle of exercise (C) and no exercise (D) mice.

Discussion

Secondary lymphedema is a common, chronic, and progressive condition that still has no curative treatment, and for which preventive strategies are currently being investigated. In this study, we have demonstrated that exercise performed before surgery and radiation in mice attenuated the development of lymphedema in terms of the severity of swelling, dermal backflow patterns on ICG imaging, and increase in skin thickness. Moreover, mice which exercised before lymphatic injury were shown to have a greater lymphatic density than those which did not exercise.

The mouse hindlimb has been used in previous studies in order to study lymphedema. However, due to spontaneous resolution of swelling, several authors have employed various strategies to create a reproducible and more long-lasting model, such as adding radiation before or after surgery [16, 22, 23] and excision of more lymph nodes including those in the inguinal area [23, 24]. In this study, combined resection of the popliteal lymph node and post-surgery irradiation with a single 20-Gy dose was able to produce a model of lymphedema which lasted at least 6 weeks and was able to show the effects of exercise performed on such model.

Mice that performed exercise prior to surgery and radiation had a lesser degree of swelling compared to mice which did not exercise. This was consistently reflected in the percentage of excess ankle diameter from the period after injury up to the end of the observation period. Percentage of excess hindpaw thickness also showed a difference between the two groups but was only significant in the last 2 weeks. From weeks 1-3, both groups showed similar PET values with an almost level trend, as opposed to the decreasing trend of the PED. A possible reason for this is that the hindpaw is also the site of injections for the blue dye and ICG, and this could mask the likely effects of exercise or even mask the effects of lymphatic dysfunction brought about by surgery and radiation. This variation within the areas of the hindlimb could also be due to regional lymph drainage failure [25]. Secondary lymphedema tend to start proximally and then proceed distally as the condition progresses [26, 27]. After week 3, the difference in PET between the exercise and no exercise groups became evident.

A possible mechanism by which exercise can attenuate lymphedema in this case may be an increase in the lymphatic capillary density, which was consistent with our immunohistochemistry

results. A higher lymphatic density may be an indication that lymphangiogenesis or the creation of new lymphatic vessels occurred due to exercise, or that previously dormant lymphatic vessels were recruited to facilitate lymphatic drainage in areas were lymphatic vessels were damaged [25]. A previous study by Hespe et al. also has exhibited similar results in which aerobic exercise was shown to increase the lymphatic vessel density in obese mice [13]. This study also demonstrated the role of exercise in decreasing perilymphatic inflammation and improving lymphatic vessel pumping [13], although these factors were not investigated in our current study. It is important to note, however, that the number of lymphatic vessels should be interpreted with caution and should be supplemented with other information supporting lymphatic vessel function. A previous study has demonstrated that lymphatic obstruction induced lymphangiogenesis which generates immature and leaky vessels, via activation of vascular endothelial growth factor (VEGF)-C/D and VEGF receptor 3 signaling and interplay of inflammatory mediators, which appear to be critical the progression of lymphedema [28]. In this current study, the lymphatic vessels of the exercise group appeared to be functional lymphatic vessels corroborated by other results such as the limb measurements which showed faster resolution of swelling over time and ICG imaging which showed splash or mild dermal backflow patterns, and collateral pathways.

Other possible mechanisms by which exercise can attenuate lymphedema is through physiological changes associated with long-term exercise including increased sympathetic outflow, increased muscular contractions which the stimulates the lymphatic pump, and increased lung ventilation which facilitates lymph return [25]. Although these factors may be more significant in the role of exercise during lymphedema when there is ongoing fluid accumulation, these could still contribute during the pre-injury phase by conditioning the lymphatic vascular system. Baseline lymphatic function has been shown to affect the outcome after lymphatic injury [12]. These, along with the increased lymphatic capillary density, may increase the therapeutic functional reserve of the trained individual, providing a safety net that would aid in avoiding or delaying compromised lymphatic function when damage occurs to the lymphatic system such as during surgery or radiation [29].

In normal mice, the efferent lymphatic flow from the hindpaw is predominantly directed into the popliteal lymph node, followed by the ischial and lumbar lymph nodes located deeply under muscles

[30]. Removal of the popliteal lymph node changes the lymphatic pattern dramatically revealing a novel lymphatic flow from the footpad to the inguinal lymph node lymph node, occurring within 1-4 weeks depending on the extent of the surgery [30, 31]. From the inguinal lymph node, lymphatic flow to the axillary lymph node may be observed but this pathway is inefficient in mice unlike in rats [32], hence was not quantified in this study. ICG imaging results revealed collateral flow to the inguinal lymph node beginning at the 3rd week for both groups, however, not all mice showed inguinal lymph node signal at the 6th week. This contrasts with previous studies wherein 100% of mice showed rerouting to the inguinal lymph node as early as the first week [30, 31]. The delayed development of collateral flow strengthens our lymphedema model and may be due to the application of radiation which affects both existing lymphatic vessels by damaging lymphatic endothelial cells, and possible new lymphatic vessels by inhibiting lymphangiogenesis [16, 22]. Comparing the 2 groups, the exercise group showed more mice with collateral flow to the inguinal lymph node at weeks 3, 5 and 6, although the difference was not statistically significant probably due to the low sample size. Nevertheless, this suggests that exercise may have facilitated the unmasking of this unconventional lymphatic pathway from the hindpaw to the inguinal lymph node, or stimulated lymphangiogenesis. Yamaji et al. have suggested that the inguinal lymph node directed flow after popliteal lymph node resection is served by pre-existing lymphatic vessels rather than newly developed vessels by lymphangiogenesis because of the short-term change in the drainage pattern, as early as 3 days after popliteal lymph node resection, as demonstrated in their study[30]. In this current study, however, collateral flow was observed during the 3rd week after lymphatic injury, hence these lymphatic vessels involved in the collateral pathways can either be new lymph vessels, or pre-existing vessels which were once dormant and recruited during lymphatic obstruction.

Moreover, our ICG imaging results showed a significant difference in the dermal backflow patterns of the two groups at weeks 1, 4 and 5 with more of the no exercise showing the more severe patterns, but the difference was also approaching significance at weeks 3 and 6 (p-value < 0.1). This gives us an idea of the quality of the lymph vessels involved. The abundance of diffuse and stardust patterns in the no exercise group may indicate lymphatic vessel leakiness and immaturity, as exercise has been shown to decrease lymphatic vessel leakiness [13]. Moreover, the mechanical stimulation

provided by the increased intralymphatic and interstitial fluid pressure during exercise may promote the development and maturation of lymphatic vessels, as demonstrated in the development of the lymphatic vasculature in the mouse embryo [33] .

Another finding of this study is the lower increase in skin thickness of the exercise group compared to the no exercise group. Lymphedema is characterized by epidermal and dermal expansion [1, 34] and this study has shown that exercise can mitigate this change. The exact mechanism of hyperkeratosis in lymphedema is still unknown, but may be related to lymph stagnation which provides growth factors for keratinocyte proliferation [35]. Hence, the limb measurements are related to the skin thickness results. The increased PED and dilated lymphatic vessels of the no exercise group reflect more lymphatic fluid stasis which could lead to increased epidermal thickness, as well as increased dermal thickness since the dermal area is where the lymphatic fluid accumulates. Moreover, the dermal thickness may also reflect adipose deposition which is another characteristic feature of lymphedema [1]. This study, however, did not distinguish adipose tissue deposition in the dermis or subcutaneous tissue areas. Only the skin thickness in the hindpaw area was included in this study because obtaining consistent cross-sectional samples in the proximal hindlimb was difficult and ran the risk of including tissue associated with the abdominal or pelvic area because of the inevitable soft tissue sliding within this region because mice are loose-skinned at this area [36].

This study has several limitations. The mechanism of how exercise affects lymphedema cannot be fully elucidated from this study. Exercise may have a multifaceted role in lymphedema and there is a need for further studies which can substantiate these factors, such as the role of exercise in lymphangiogenesis or lymphatic reorganization, effect on lymphatic vessel pump and pathologic changes such as inflammation and adipose deposition. Our ICG imaging results were limited to observational findings. Dynamic studies can provide more information on lymphatic flow and pumping activity. Moreover, our study was limited to exercise before lymphatic injury. It would be more significant to investigate the effect of exercise at other time points, in particular after lymphatic injury and compare to pre-injury exercise.

Conclusion

This study showed that in mice hindlimb model of lymphedema, pre-injury exercise led to a lesser degree of swelling, less severe dermal backflow patterns on ICG imaging, lesser increase in skin thickness and a greater density of lymphatic vessels. This suggests that exercise during prehabilitation may be a potential prophylactic strategy that may attenuate the development of lymphedema after surgical and radiological ablation of lymphatics.

Reference

1. Li, C.Y., R.P. Kataru, and B.J. Mehrara, *Histopathologic Features of Lymphedema: A Molecular Review.* Int J Mol Sci, 2020. **21**(7).
2. Fu, M.R., et al., *Psychosocial impact of lymphedema: a systematic review of literature from 2004 to 2011.* Psychooncology, 2013. **22**(7): p. 1466-84.
3. Cormier, J.N., et al., *Lymphedema beyond breast cancer: a systematic review and meta-analysis of cancer-related secondary lymphedema.* Cancer, 2010. **116**(22): p. 5138-49.
4. DiSipio, T., et al., *Incidence of unilateral arm lymphoedema after breast cancer: a systematic review and meta-analysis.* Lancet Oncol, 2013. **14**(6): p. 500-15.
5. Hayes, S.C., et al., *Lymphedema following gynecological cancer: Results from a prospective, longitudinal cohort study on prevalence, incidence and risk factors.* Gynecologic Oncology, 2017. **146**(3): p. 623-629.
6. Silver, J.K. and J. Baima, *Cancer prehabilitation: an opportunity to decrease treatment-related morbidity, increase cancer treatment options, and improve physical and psychological health outcomes.* Am J Phys Med Rehabil, 2013. **92**(8): p. 715-27.
7. Johansson, K. and S. Hayes, *A historical account of the role of exercise in the prevention and treatment of cancer-related lymphedema.* Lymphology, 2020. **53**(2): p. 55-62.
8. Schmitz, K.H., et al., *Physical Activity and Lymphedema (the PAL trial): assessing the safety of progressive strength training in breast cancer survivors.* Contemp Clin Trials, 2009. **30**(3): p. 233-45.
9. Schmitz, K.H., et al., *Weight Lifting for Women at Risk for Breast Cancer-Related Lymphedema A Randomized Trial.* Jama-Journal of the American Medical Association, 2010. **304**(24): p. 2699-2705.
10. Singh, B., et al., *Systematic Review and Meta-Analysis of the Effects of Exercise for Those With Cancer-Related Lymphedema.* Archives of Physical Medicine and Rehabilitation, 2016. **97**(2): p. 302-315.
11. Harris, S.R. and S.L. Niesen-Vertommen, *Challenging the myth of exercise-induced lymphedema following breast cancer: A series of case reports.* Journal of Surgical Oncology, 2000. **74**(2): p. 95-98.
12. Hespe, G.E., et al., *Baseline Lymphatic Dysfunction Amplifies the Negative Effects of Lymphatic Injury.* Plast Reconstr Surg, 2019. **143**(1): p. 77e-87e.
13. Hespe, G.E., et al., *Exercise training improves obesity□related lymphatic dysfunction.* The Journal of Physiology, 2016. **594**(15): p. 4267-4282.
14. Liu, Z., et al., *Moderate-Intensity Exercise Affects Gut Microbiome Composition and Influences Cardiac Function in Myocardial Infarction Mice.* Front Microbiol, 2017. **8**: p. 1687.

15. Ferreira, J.C., et al., *MAXIMAL LACTATE STEADY STATE IN RUNNING MICE: EFFECT OF EXERCISE TRAINING.* Clinical and Experimental Pharmacology and Physiology, 2007. **34**(8): p. 760-765.
16. Wang, Z., et al., *Radiation Inhibits Lymph Drainage in an Acquired Lymphedema Mouse Hindlimb Model.* Lymphat Res Biol, 2020. **18**(1): p. 16-21.
17. Liao, S.F., et al., *The efficacy of complex decongestive physiotherapy (CDP) and predictive factors of lymphedema severity and response to CDP in breast cancer-related lymphedema (BCRL).* Breast, 2013. **22**(5): p. 703-706.
18. Kim, M., et al., *Identification of prognostic risk factors for transient and persistent lymphedema after multimodal treatment for breast cancer.* Cancer Res Treat, 2016. **48**(4): p. 1330-1337.
19. Johansson, K. and E. Branje, *Arm lymphoedema in a cohort of breast cancer survivors 10 years after diagnosis.* Acta Oncol, 2010. **49**(2): p. 166-173.
20. Allen, R.J., Jr. and M.H. Cheng, *Lymphedema surgery: Patient selection and an overview of surgical techniques.* J Surg Oncol, 2016. **113**(8): p. 923-931.
21. Yamamoto, T., et al., *Characteristic indocyanine green lymphography findings in lower extremity lymphedema: the generation of a novel lymphedema severity staging system using dermal backflow patterns.* Plast Reconstr Surg, 2011. **127**(5): p. 1979-1986.
22. Dai, T.T., et al., *A refined mouse hind limb model of secondary lymphedema.* International Journal of Clinical and Experimental Medicine, 2016. **9**(4): p. 7565-7572.
23. Wiinholt, A., et al., *A Revised Method for Inducing Secondary Lymphedema in the Hindlimb of Mice.* J Vis Exp, 2019(153).
24. Nakajima, Y., et al., *Near-Infrared Fluorescence Imaging Directly Visualizes Lymphatic Drainage Pathways and Connections between Superficial and Deep Lymphatic Systems in the Mouse Hindlimb.* Sci Rep, 2018. **8**(1): p. 7078.
25. Lane, K., D. Worsley, and D. McKenzie, *Exercise and the Lymphatic System.* Sports Medicine, 2005. **35**(6): p. 461-471.
26. Fujiyoshi, T., et al., *Pathological Changes in the Lymphatic System of Patients with Secondary Lower Limb Lymphedema Based on Single Photon-Emission Computed Tomography/Computed Tomography/Lymphoscintigraphy Images.* Lymphat Res Biol, 2021.
27. Mikami, T., et al., *Pathological changes in the lymphatic system of patients with secondary upper limb lymphoedema.* Sci Rep, 2019. **9**(1): p. 8499.
28. Ogata, F., et al., *Excess Lymphangiogenesis Cooperatively Induced by Macrophages and CD4(+) T Cells Drives the Pathogenesis of Lymphedema.* J Invest Dermatol, 2016. **136**(3): p. 706-714.
29. Dolan, L.B., K.N. Lane, and D.C. McKenzie, *Is there enhanced lymphatic function in upper body trained females?* Lymphat Res Biol, 2008. **6**(1): p. 29-38.

30. Yamaji, Y., et al., *Development of a mouse model for the visual and quantitative assessment of lymphatic trafficking and function by in vivo imaging.* Sci Rep, 2018. **8**(1): p. 5921.
31. Blum, K.S., et al., *Dynamics of lymphatic regeneration and flow patterns after lymph node dissection.* Breast Cancer Res Treat, 2013. **139**(1): p. 81-6.
32. Harrell, M.I., B.M. Iritani, and A. Ruddell, *Lymph node mapping in the mouse.* J Immunol Methods, 2008. **332**(1-2): p. 170-4.
33. Planas-Paz, L., et al., *Mechanoinduction of lymph vessel expansion.* Embo Journal, 2012. **31**(4): p. 788-804.
34. Jiang, X., et al., *Decreased lymphatic HIF-2alpha accentuates lymphatic remodeling in lymphedema.* J Clin Invest, 2020. **130**(10): p. 5562-5575.
35. Domaszewska-Szostek, A., M. Zaleska, and W.L. Olszewski, *Hyperkeratosis in human lower limb lymphedema: the effect of stagnant tissue fluid/lymph.* J Eur Acad Dermatol Venereol, 2016. **30**(6): p. 1002-8.
36. Jorgensen, M.G., et al., *Quantification of Chronic Lymphedema in a Revised Mouse Model.* Ann Plast Surg, 2018. **81**(5): p. 594-603.

Korean Abstract

배경 및 목적 림프부종은 암 치료 후 흔하지만 고통스러운 후유증이다. 아직까지 완전한 치료법이 없기 때문에 예방은 그 관리의 필수적인 부분이다. 본 연구는 림프절 절제술 및 방사선치료에 의한 손상 전 운동이 마우스 하지 림프부종에 미치는 영향을 알아보고자 하였다.

방법 BALB/c 마우스는 운동군(n=7)과 비운동군(n=7)에 임의로 배정되었다. 운동군은 중등도 강도의 트레드밀 운동을 4 주동안 1 주일에 5 일, 각 30 분간 시행하였고, 비운동군은 같은 시간 동안 고정된 트레드밀에 놓여졌다. 림프부종은 편측 오금 림프절 절제 후 20 Gy 의 하지 방사선 조사를 통해 유도되었다. 발목 직경과 발의 두께는 매주 6 주동안 캘리퍼 측정을 통해 얻어졌으며, 비손상측 하지와 비교하여 초과한 발목 직경(PED)과 발의 두께(PET)값은 백분율로 계산되었다. 인도시아닌그린(indocyanine green) 림판지오 영상은 피부 역류 패턴(dermal backflow)을 시각화 하기 위해 매주 시행되었다. 상피 두께와 림프관 밀집도를 평가하기 위해 손상 6 주 후 조직병리학적 분석을 실시하였다.

결과 운동군은 비운동군에 비해 손상 후 3, 4, 5, 6 주 차에 유의미하게 낮은 PED 값을 보였으며, 손상 후 5, 6 주 차에는 유의미하게 낮은 PET 를 보였다. 인도시아닌그린 촬영 결과, 손상 후 1, 4, 5 주에 비운동군에서 더 심각한 스타더스트(stardust)와 확산된(diffuse) 패턴을 보였다. 헤마톡실린과 에오신 슬라이드에서 운동군은 상피와 진피 두께에서 유의미하게 낮은 증가를 보였다. 항- LYVE-1 을 이용한 면역조직화학적 염색에서는 운동군에서 비운동군보다 더 높은 림프관 밀집도를 보였다.

결론 림프 손상 전 운동은 림프절 절제술 및 방사선조사에 의해 유도되는 마우스 하지의 이차성 림프부종의 발달을 약화시킬 수 있다. 이 결과는 인간 림프부종의 예방적 재활운동 역할에 영향을 미칠 수 있다.

색인단어 신체적 활동, 전재활운동, 림프관형성, 이차성 림프부종