Doctor of Philosophy

Effect of hyaluronidase for regeneration of vascularized lymph node flap transfer in the experimental animal study

The Graduate School

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Department of Plastic surgery

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Author Linhai Chen

ABSTRACT

Background and purpose: Lymphedema is a chronic, debilitating and consumptive condition of an overwhelmed lymphatic system, which affects up to 250 million individuals worldwide. Currently, although there is still no cure for lymphedema we have made some breakthroughs in surgical treatment and medication. Physiological surgical treatment for lymphedema, especially Vascularized lymph node flap (VLNF) transfer, have been proven to improve the clinical efficacy in lymphedema and promote functional recovery of the affected area, as well as reduce the incidence of cellulitis by reversing the pathological process of lymphedema. However, the transfer flap could form fibrous adhesions with the surrounding tissues and extracellular matrix deposition resulting in blocked lymphangiogenesis and lymphatic fluid obstruction, which is the biggest issue affecting the efficacy of VLNF transfer. Furthermore, hyaluronidase not only enhances lymphangiogenesis in lymphedema limbs but also reduces the level of tissue fibrosis. The purpose of this study was to evaluate the efficacy of VLNF transfer combined with hyaluronidase injection.

Materials and methods: The experiment was designed to utilize 34 male adult Sprague-Dawley (SD) rats weighing 250–300 g. First, all rats were established in a model of VLNF transfer for lymphedema. Then, rats were categorized into 2 groups, the experimental group, and the control group, based on whether or not hyaluronidase was injected. Each group is divided into three parts: In part I, 6 rats were recorded weekly for forelimb volume using photographic post-software analysis and observation of lymphatic drainage patterns in NIR indocyanine green imaging. In part II, 1 rat was sacrificed every 4 weeks to redissect the

surgical area under the microscope to observe the level of lymphangiogenesis and the degree of surrounding fibrosis. In addition, the velocity of lymphatic transport was calculated by NIR indocyanine green imaging system every two weeks in this group of rats. In Part III, 2 rats were sacrificed every 3 weeks for pathological examination to quantify lymphangiogenesis and limb fibrosis. After 12 weeks of the experiment, all rats were euthanized.

Results: In the experimental group, the change of forelimb volume was higher than that in the control group. Moreover, with the progress of the experiment, the linear drainage pattern displayed by ICG accounted for a higher proportion in the experimental group, and the upward trend was more obvious. Similarly, in the comparison of lymph node transfer velocity, the data of the experimental group was much higher than that of the control group and was almost close to the normal lymph node metastasis rate at the later stage of treatment. What's more, the pathological results showed that the content of lymphatic vessels was more, while the content of collagen fibers was less in the experimental group.

Conclusion: In animal experiments, hyaluronidase injection can optimize the therapeutic effect of VLNF transfer in lymphedema. This is because the combination treatment promotes lymphangiogenesis and reduces fibrosis around the VLNF. Despite the efficacy, its clinical application remains to do further study.

Keywords: Vascularized lymph node flap; Hyaluronidase; Lymphedema; Rat; Treatment

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LIST OF ABBREVIATIONS

ALNF	Axillary lymph node flap
BLNF	Brachial lymph node flap
LN	Lymph node
SD	Sprague–Dawley
HLD	Hyaluronidase
VLNF	Vascularized lymph node flap
LVA	Lymphovenous anastomosis
ICG	Indocyanine green
NIRF	Near infrared fluorescence
VEGF	Vascular endothelial growth factor
LT	Leukotrienes
IL	Interleukin
TGF-β1	Transforming growth facter-β1
IGF	Insulin-like growth factors
HGF	Hepatocyte growth factor
PBS	Phosphate buffer solution
LYVE-1	Lymphatic Vessel Endothelial Hyaluronic Acid Receptor-1
MT	Masson's Trichrome

1.INTRODUCTION

Lymphedema represents a common, progressive and chronic disorder that results from primary or acquired dysfunction of the lymphatic system, which occurs essentially as an imbalance of lymphatic loading and transport capacity result in the accumulation of high-protein interstitial fluid. Patients with lymphedema have a low morbidity rate of roughly 1 in 30, affecting 250 million people worldwide [1-3]. Lymphedema is classified as primary or secondary depending on its etiology, in general, most cases are secondary, which accounts for approximately 99% of cases. Primary lymphedema is very rare, Idiopathic lymphedema was found in 70% of these patients, and identifiable genetic mutations were found in the remaining 30%, many of which involved the signaling pathway for VEGF-C [4-5]. In terms of secondary, the common etiology is a parasitic infection in developing countries, mainly through indirect or direct factors blocking the lymphatic vessels, while cancer-associated iatrogenic factors in developed countries, especially for surgical treatment of breast and gynecologic cancers, including lymph node dissection and adjuvant radiotherapy [6-7]. Lymphedema is typically pathologically characterized by chronic local inflammation, remodeling and fibrosis of the extracellular matrix, deposition of adipose tissue, and progressive sclerosis due to prolonged stasis of lymph fluid. The process does not occur independently but rather resembles an inline reaction that creates a waterfall effect [8-9].

Despite a deeper understanding of the pathogenesis and progression of lymphedema, there is still no cure [1]. Therefore, our treatment goals are to improve the impact of the bulky extremity on life, reduce the rate of hospitalization of patients, and reduce the incidence of

complications, including lymphangitis, cellulitis, and dengue [2]. Regular treatments include pharmacotherapy, complete decongestive therapy, and surgical treatment. Although some medicines, such as diuretics, coumarin, steroids, and Ketoprofen, have demonstrated efficacy in reducing swelling in experiments with mouse models of lymphedema, and no evidence that they can manage or prevent lymphedema in the long term [3]. Moreover, the side effects of the drugs may exacerbate limb swelling. In addition, complete decongestive therapy has become the mainstay of initial treatment for patients with lymphedema as a non-surgical or conservative measure, which is a multimodality approach including bandaging, exercise, manual lymphatic drainage, and skincare [10]. However, in the field of surgical treatment of lymphedema, significant progress has been made in recent years, both for the study of treatment mechanisms and the improvement of surgical techniques. Options for surgical therapies are divided into two categories: physiologic and non-physiologic [11]. Physiologic options aim to promote improved lymphatic flow in a manner, LVA and VLNF transfer, by increasing the number of lymph node and lymphatic vessels access patency, whereas non-physiologic options, like ablation procedures, is simply debulk areas of lymphedema to reduce morbidity [12-13].

VLNF transfer, as a physiologic lymphatic procedure, has been shown to improve the clinical presentations in lymphedema and promote functional recovery of the affected area, as well as reduce the incidence of cellulitis by reversing the pathological process of lymphedema, additionally, lessen the burden of non-surgical treatment by reducing reliance on compression [14]. From the therapeutic principle, VLNF transfer allows for the reintroduction of interstitial fluid into the venous and lymphatic systems by replacing lost or damaged lymphatic units,

lymph nodes, and lymphatic vessels, with healthy and functioning lymphatic units [15]. In the early practice, since 1979, many animal experiments and clinical studies have demonstrated the positive results of VLNF transfers and found that lymphatic vessels have an enormous regenerative capacity after tissue transfer and spontaneously reconnect with the surrounding existing lymphatic vessels [16]. Furthermore, there have been some significant advances that have made VLNT increasingly popular for instance，Becker et al. published the VLNF can be harvested for postmastectomy lymphedema from the lower abdominal wall and transferred into the axilla. Tobbia et al, found better clinical outcomes with vascularized lymphatic grafts compared to non-vascularized lymphatic grafts and Blum et al. found that larger fragments were more easily integrated into the lymphatic system than smaller lymph node section [17]. Although the steps for VLNF transfer are clear, the detailed mechanism of VLNF transfer remained controversial. One proposed mechanism is that VLNF acts as a “pump” that interstitial fluid was absorbed by the transferred lymph nodes within the flap and flowed into the donor's vein via a gradient between venous outflow and arterial inflow. The other proposed mechanism is that VLNF acts as a “Bridge” that interstitial fluid enters the venous system through a lymphatic pathway that is formed by the spontaneous anastomosis of lymphangiogenesis between the donor and recipient. Either mechanism must ensure the reduction of donor fibrosis and the complete release of scar tissue in the recipient area [18]. Nevertheless, there are inevitable that the transfer flap must form fibrous adhesions with the surrounding tissues and extracellular matrix deposition resulting in blocked lymphangiogenesis

and lymphatic fluid obstruction, which is the biggest problem and challenge affecting the efficacy of VLNF transfer [14].

How to address this problem is the focus of our study. Nevertheless, there are only a few studies in the previous literature on combined VLNF transfer for lymphedema [19-21]. For instance, we all know that the VEGFR3/VEGF-C-D axis is a reliable target for enhancing lymphangiogenesis and has been shown to promote lymphatic vessel regeneration in VLNFs. Additionally, a array of different chemokines and cytokines, such as TGF-β1, IGF, and HGF, regulate lymphatic vessel remodeling and valve development by directly or indirectly affecting the VEGFR3/VEGF-C-D axis [19]. But there is a potential problem that the tumor microenvironment may be activated by a similar proangiogenic effect of VEGF-C and others [20]. Moreover, some literature found that progenitor cells or stem cells can be used to regenerate lymphoid tissue, such as adipose-derived mesenchymal stem cells and bone marrow stem cells, to proliferate lymph endothelial cells at the initial stage of cell differentiation [21-22]. Again, this would not be an appropriate adjuvant therapy combined with VLNF transfer because stem cell injection requires complex procedures and a high cost of treatment. Instead, some studies found that Hyaluronidase (HLD) not only reduces swelling volume but also promotes lymphangiogenesis in lymphedema limbs [23]. In addition, HLD can regulate extracellular matrix protein and glycosaminoglycan content to change the interstitial environment [24]. Therefore, we propose that HLD injection could be used as a combination therapy to optimize the efficacy of VLNF transfer for lymphedema.

We have found that there is a trend towards combining VLNF transfer with medical therapy, and studies have shown that VEGF-C injections around VLNF, either by gel-based or adenoviral drug delivery, can further improve treatment outcomes [25-26]. Hence, we believe that combined VLNF transfer and hyaluronidase injections are feasible. We designed this animal study to investigate whether hyaluronidase injection optimizes the effect of VLNT transfer for lymphedema.

2.MATERIALS AND METHODS

2.1. Animals

The study utilized 34 Sprague–Dawley (SD) rats of male and weighing 250-300g. They were all purchased from the Animal Center of ASAN Research Institute. All of the SD rats were adaptively fed for 1 week preoperatively, allowed to drink water and feed ad libitum, and maintained under SPF conditions. The procedures related to animal experiments were performed on specific premises with specifications. All operations were reviewed and approved by the Ethics Committee of the Asan medical center. At the end of the experiment, the anesthetized rats were sacrificed by exposing animals to CO2 for 7 minutes. The euthanasia protocol was following the American Veterinary Medical Association guidelines for the euthanasia of animals.

2.2. Reagents and Solution configuration

The reagents used in the experiment are as follows: Normal saline was purchased from JW Pharmaceutical Co. (Seoul, Korea). 75% medical alcohol was purchased from Health maximum Co. (Jecheon, Korea). Iodophor was purchased from Health maximum Co. (Jecheon, Korea). Formalin was purchased from Hisko (Gunpo, Korea). Depilatory cream was purchased from Nair, Church & Dwight Co (New Jersey, USA). Hyaluronidase (HLD) was purchased from Daehan New Pharm Co. (Seoul, Korea). Isoflurane was purchased from JW Pharmaceutical Co. (Seoul, Korea). Zoletil was purchased from Virbac (Seoul, Korea). Methylene blue was purchased from Duksan (Sinworo, Korea). Indocyanine green (ICG) was

purchased from Dongindang Pharm (Siheung, Korea). Ibuprofen was purchased from Daehan New Pharm Co. (Seoul, Korea). Trichrome stain (MASSON) Kit (cat. no. ab150686; Abcam) was purchased from Sigma-Aldrich (Sigma, USA). Antibody LYVE-1 (cat. no. ab14917; Abcam) was purchased from Abcam, Inc. (Cambridge, USA). Hematoxylin and Eosin Staining Kit (Beyotime) was purchased from Abcam, Inc. (Cambridge, USA).

Common solutions made in this experiment include 1. Methylene blue solution: 1g methylene blue powder is dissolved in 50ml distilled water.2. ICG solution: 0.1 mL/kg, dissolved in saline to a ratio of 2.5 mg/mL. 3. Formalin solution: 10ml of 40% formaldehyde liquid is mixed with 90ml of distilled water to obtain 4% formaldehyde, that is, 10% formalin 4. HLD solution: Each bottle was injected with 0.5ml normal saline with a concentration of 3000 UI/ml 5. Anesthetic solution: 5 mL sterile water for injection should be added to each vial. Each milliliter of the resulting solution will contain 100 mg total Zoletil for Injection.

2.3. Animal experiment model surgery

Before the operation, all rats were further anesthetized with Zoletil after being induced by isoflurane gas in a concentration of 4%. Then, the body hair and fur were shaved with electric clippers and depilatory cream. The surgical area was located in the right forelimb. After disinfecting the area with 75% medical alcohol, approximately 0.05 mL methylene blue solution was subcutaneously injected into the palmar side of the hand of the rats and the injection site was gently massaged for approximately 30 seconds.

After the preparation was in place, the rats were supine on the operating table and the microsurgical operations were as follows: Step 1: A circumferential incision was designed through the axillary inferiorly and shoulder joints superiorly, the dorsal incision must exceed the projection point of the brachial lymph nodes on the body surface, and the ventral incision is located in the transition area between the forelimb and the chest (Figure. 1A). Step 2: A dorsal incision to expose the brachial lymph node, which is located between the triceps brachii and latissimus dorsi. Once the blue-stained lymph node is found, surrounded by loose adipose tissue, the lower border of the tissue is first sharply peeled off, then the tissue is raised to separate the deep surface, then the tissue is raised to bluntly separate the deep surface until a main vascular pedicle is observed, which is cauterized with a high-frequency electrotome Generally, there is a group of small vascular pedicles on the medial and lateral of the tissue that has to be cauterized. Finally, with the blood supply cut off, the brachial lymph nodes are removed with scissors, and the surrounding lymphatics are excised together (Figure. 1B). Step 3: axillary lymph node flap is concealed in the deep surface of the pectoralis major. There are two dissecting approaches: one is to incise the muscle fibers in parallel at the superficial side of the muscle (the incision is usually about 5mm inward from the lateral edge of the muscle); the other is to cut the muscle fibers in parallel after exposing the deep side of the muscle by a retractor. Considering the simplicity of the operation, we are more inclined to choose the former. Much more, the branches of the brachial plexus could be gently stripped above the flap to reduce iatrogenic damage. Immediately the blue-stained lymph node was observed, the medial edge of the flap is first sharply peeled off, then the flap is elevated to bluntly separate the deep

surface, followed by the lateral edge. Usually, there are two small vascular pedicles at the distal of the flap that has to be cauterized. Finally, after the flap was completely lifted, the pedicle was carefully skeletonized and the flap was placed in situ (Figure. 1C). Step 4: Suturing is performed in the fashion of folding to prevent reconnection of intradermal lymphatic vessels. It should be noted that the skin incision must be cauterized circumferentially and subcutaneously free of the incision by 1-2 mm before suturing (Figure. 1D). All rats were operated on by the same surgeon. Ibuprofen (1mg/kg) was injected intramuscularly immediately after the operation.

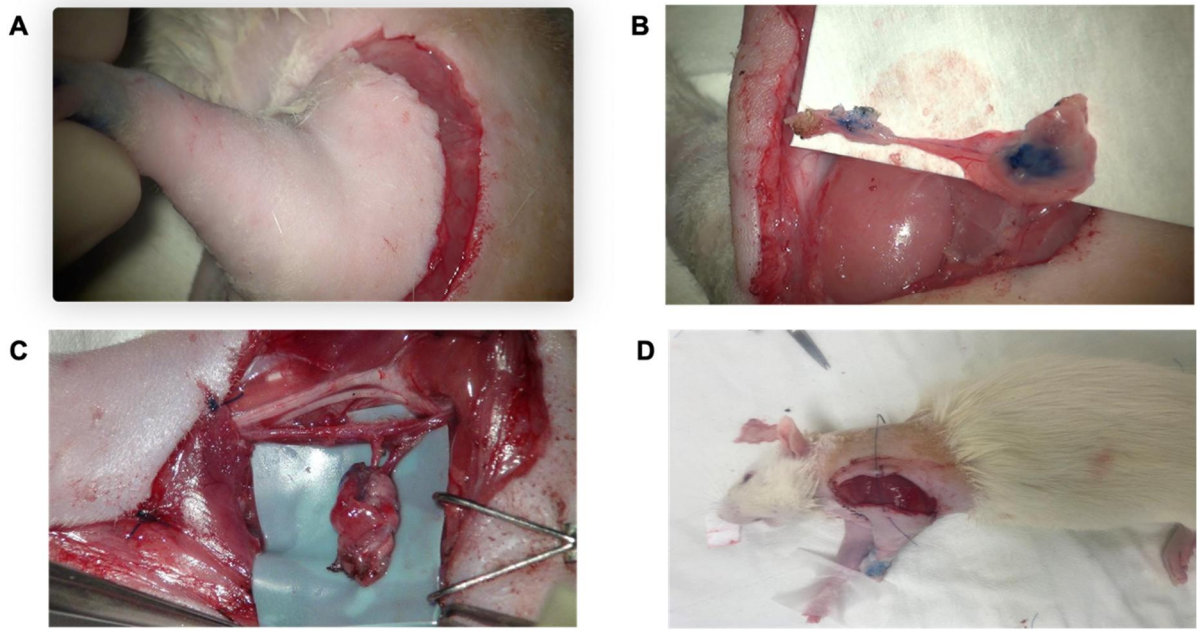


Figure 1. Procedure of animal model surgery.

(A) Circular incision. (B) Remove brachial axillary LN. (C) Elevate the axillary LN flap. (D) Folding suture.

2.4. Radiation

Four days after recovery from the surgery, all of the rats were irradiated at a single dose of 20 Gy using an X-Rad 320 device (Precision X-Ray, USA). Specifically, the rats were anesthetized with 4% isoflurane gas before irradiation, placed in the prone position, and then only the whole right forelimb was exposed under the protection of a special lead template. To perform this with a lower risk of morbidity, a total cumulative dose of 20 Gy was delivered in 10 fractions of 2.0 Gy at a rate of 1 Gy/min. Finally, the surgical area was disinfected at the points with iodophor and then were put in the cage.

2.5. Experimental protocol

After radiation, rats were acclimated for 7 days before being subjected to the random grouping of cohorts. Rats were divided into the experimental group (n=17) and the control group (n=17). Rats in the experimental group were subcutaneously injected 0.5 mL HLD solution at a concentration of 3000 IU/ mL every week, evenly divided into two equal parts, and injected into the subaxillary and dorsal proximal forelimb region. The control group received the same dose of saline at the same site. Each group is divided into three parts: In part I, 6 rats were recorded weekly for forelimb volume utilizing photographic post-software analysis and observation of lymphatic drainage patterns in near-infrared ICG imaging. In part II, 1 rat was sacrificed every 4 weeks to redissect the surgical area under the microscope to observe the situation of lymphangiogenesis and fibrosis around the ALNF. In addition, the velocity of lymphatic transport was calculated by NIR indocyanine green imaging system every

two weeks in this group of rats. In Part III, 2 rats were sacrificed every 3 weeks for pathological examination to quantify lymphangiogenesis and fibrosis around the ALNF.

2.6. Forelimb volume measurement

Rats were placed in the prone position with the forelimbs naturally flexed on the plane in a completely relaxed state after anesthesia. Pictures were taken and two-dimensional data in forelimbs were measured and analyzed in the Image-J software (Image J 1.48v; NIH, Bethesda, Md.) through the proportion of reference objects, Including the diameter of the wrist, the diameter of the elbow, and the distance between the wrist and elbow (Figure. 2A and 2B). The volume of the forelimbs was calculated from the circumference measurements of the frustum and the cylinder model methods.

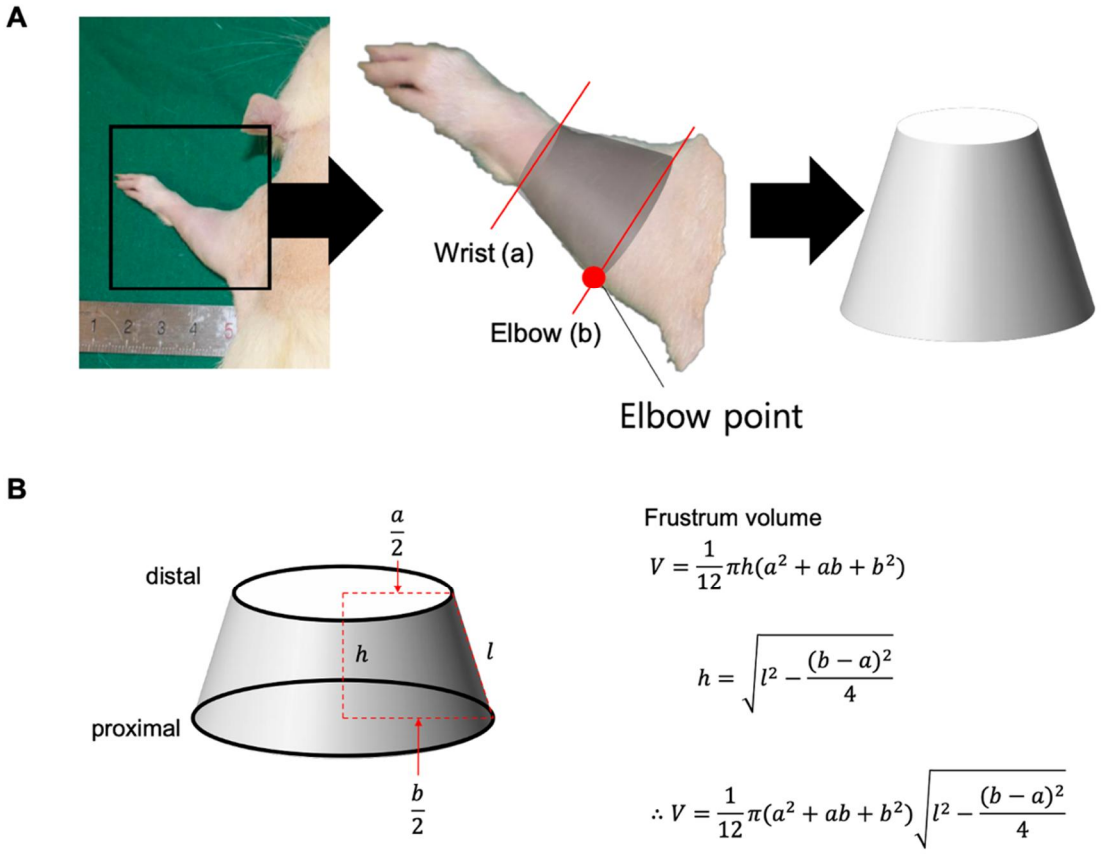


Figure 2. Forelimb volume measurement.

(A) Schematic diagram of volume measurement. (B) Models and formulas for volumetric measurement.

2.7. NIRF-ICG lymphatic imaging

At every week of follow-up, the hair on the forelimbs of rats was shaved and depilated with a depilatory cream after the rats were anesthetized with 4% isoflurane gas. A volume of 6 μL ICG was injected intradermally into the right palm with 34-gauge needles. Images were acquired in real-time, lasted for at least 15-minutes, by photo and video using a NIRF camera system (ISCAN, Burlington, MA). In the forelimbs of rats, the drainage pattern of ICG in lymphedema can be divided into linear (Figure. 3A), splash (Figure. 3B), stardust (Figure. 3C), diffuse (Figure. 3D) based on the severity of lymphedema.

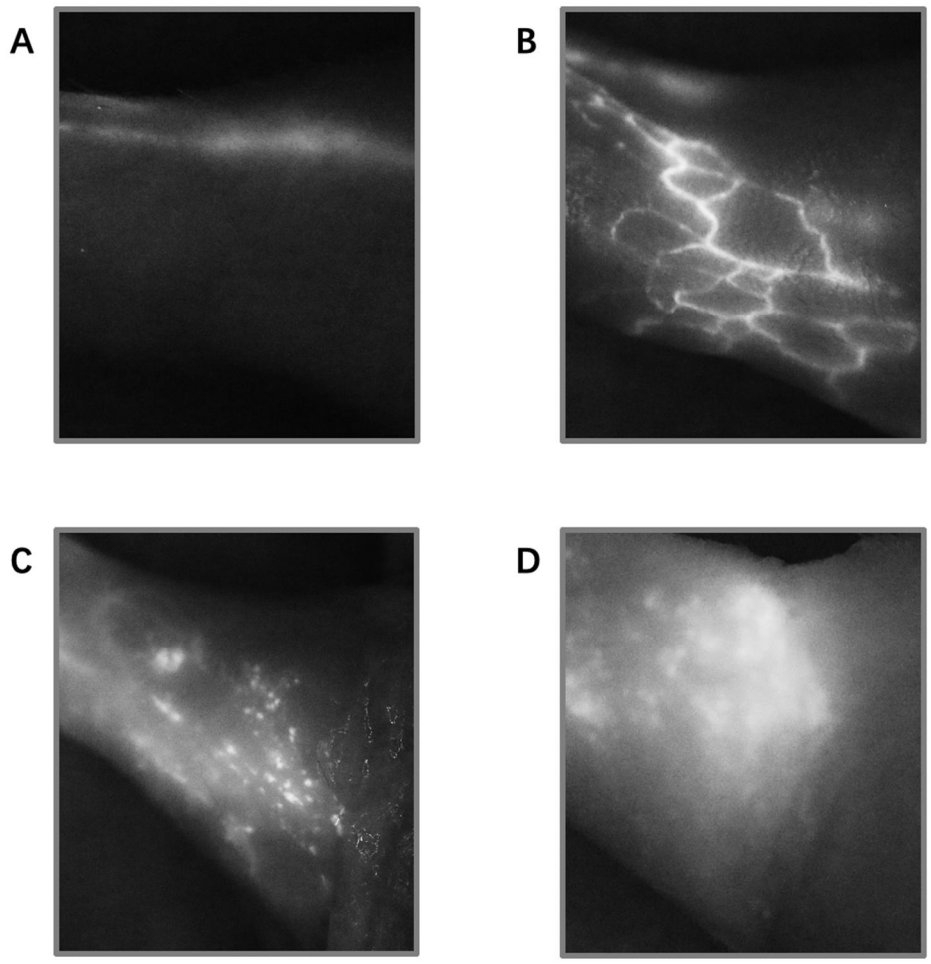


Figure 3. ICG drainage patterns.

(A) Linear mode. (B) Splash mode. (C) Stardust mode. (D) Diffuse mode.

2.8. Redissection

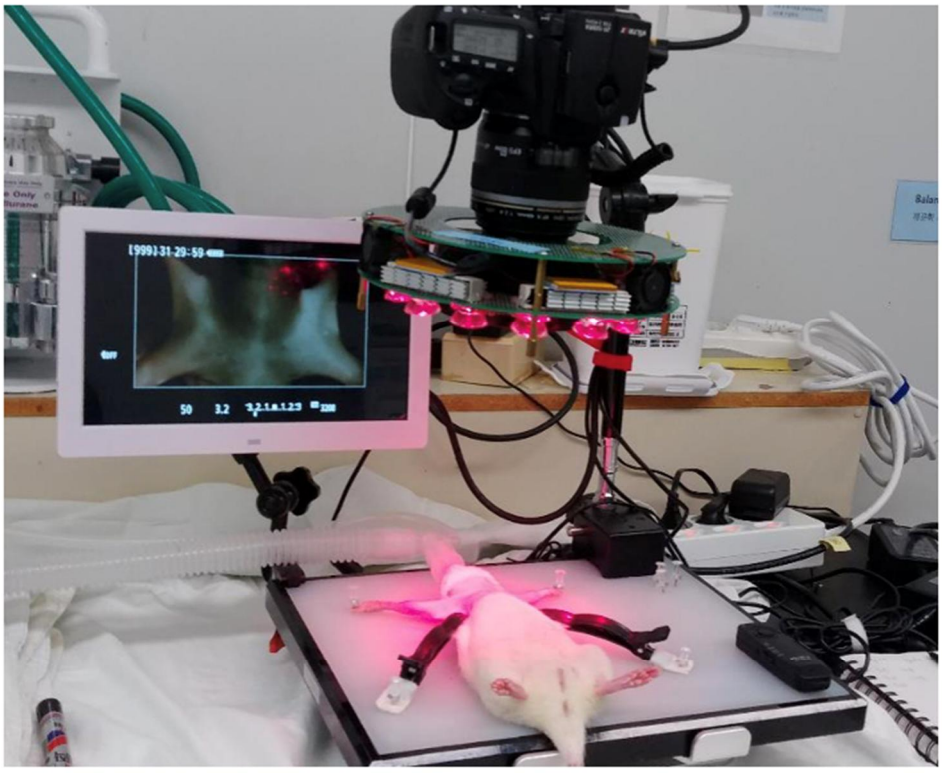
The operation of redissection is similar to the surgical procedure described above. After complete anesthesia, an axillary incision (1.0–1.5 cm) was made along the original circumferential incision. Following blunt dissection through the exposed subcutaneous fat and

muscle, via a previously selected surgical approach, an adipose flap was identified and adhered to the surrounding connective tissue. Carefully microdissect the peri-flap tissue and observe the condition of fibrous adhesions and lymphangiogenesis under a 4x surgical microscope.

2.9. Velocity of ICG drainage into transferred VLNF

We defined the lymphatic fluorescence intensity over time as the velocity of lymphatic drainage. By measuring the fluorescence intensity of axillary lymph nodes over 15 minutes using the customized ICG lymphangiography device (Figure. 4A), it should be noted that the skin and tissue covering the lymph nodes should be removed to detect accurate changes in ICG intensity. We used an asymptotic regression function to quantify the dynamic results since the intensity increases exponentially and the limit of growth is predicted to be caused by the accumulation of ICG molecules in the lymph nodes. The coefficient a is the maximum value, b is the y-intercept value, and c is the growth rate (Figure. 4B).

A



B

$$Y = a - (a - b)\exp(-cX)$$

Figure 4. Velocity of ICG drainage into transferred VLNF.

(A) The customized ICG lymphangiography device. (B) Formula of asymptotic regression function

2.10. Histological analysis

For LYVE-1 staining, The harvesting samples, rinsed with pre-cooled PBS, were fixed in 4% paraformaldehyde (Sigma) for 48 hours and washed with 70% medical alcohol, followed by dehydration with ethyl alcohol, permeabilization, impregnation with soft paraffin, hard paraffin embedding, and cutting into 4 μm sections.After antigen extraction using citrate buffer and paraffin removal, samples were blocked with 10% bovine serum albumin and stained with rabbit anti-mouse lymphatic vascular endothelial receptor (LYVE)-1 antibody (AngioBio) and biotin anti-rabbit secondary antibody (Vector Labs). Before the tissues were counterstained with hematoxylin, the Vectastain Elite ABC system for peroxidase and DAB as chromogens were utilized (Vector Labs). From the images at 20× magnification (Model BX40; Olympus, Tokyo, Japan), LYVE-1 expression in 3 different fields in each section was examined. Next, Lymphatic vessel number were measured and analyzed using Image-J software (NIH, Bethesda, MD).

For Masson's Trichrome Staining, a trichrome stain kit was used following the product introduction. The slides were washed with xylene at least twice for approximately 10 minutes to remove the paraffin before washing with deionized water, followed by staining the deparaffinized sections in Weigert's Iron Hematoxylin Solution for 10 min and washing in running warm water for 5 min. Then, sections were stained in Biebrich scarlet-acid fuchsin solution for 15 minutes and rinsed briefly in deionized water. Next, place slides in 1% Phosphomolybdic Acid Solution for 10 minutes, transferred into aniline blue solution for 5 minutes, and 1% Acetic Acid for 1 min, respectively. Finally, dehydrate samples in 95 percent

and 100 percent alcohol, clarify them in xylene, and mount them. Images of 3 different areas of staining in each section were randomly selected from the magnified 10x images (BX40 type; Olympus, Tokyo, Japan). Next, the extent of fibrosis was quantified by measuring the fiber area using Image-J software (NIH, Bethesda, MD).

2.11. Statistical analysis

Data are expressed as the mean ± standard error of the mean. Analyses were conducted using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA) and SPSS ver. 19 software (Chicago, IL, United States). One-way ANOVA or two-way ANOVA was used to detect significant differences. The statistical significance was set at p-Values less than 0.05.

3.RESULTS

3.1. Changes in forelimb volume in rats

In terms of forelimb volume in rats, we followed up for a total of 12 weeks. In the control group, the forelimb volumes were 1548.87±22.48, 1729.34±235.18, 1706.96±100.33, 1637.02±98.69, 1566.90±52.84, 1540.67±30.61, 1499.95±28.81, 1452.43±45.49, 1459.91±27.84, 1482.39±58.24, 1504.83±61.62 and 1514.44±49.28, while in the experimental group the forelimb volumes were 1515.42±19.35, 1695.02±19.35, 1532.57±34.87, 1503.22±62.10, 1455.66±116.38, 1425.43±51.24, 1425.64±31.33, 1415.55±58.73, 1430.43±47.76, 1470.33±45.96, 1486.22±31.00 and 1502.06±39.93 (Figure. 5A). In the comparison of both groups, the control group had a larger volume than the experimental group, but only the data at weeks 3, 4, 5, 6, 7 and 8 were statistically different ($p<0.05$). The Significant p-values were 0.032, 0.04, 0.019, 0.011, 0.024 and 0.036, respectively. In addition, there was no statistical difference in the volume of the forelimbs of the rats in the two groups before surgery, and there was no statistical difference in the change in body weight between the two groups during the follow-up (Figure. 5B).

Overall, based on the reliable animal model of simulated VLNF transfer for lymphedema, the experimental group began to show a significant decrease in forelimb volume after 2 weeks of treatment with HLD injection. And this statistical difference was maintained in the subsequent treatment until 8 weeks of treatment. The forelimb volumes of the rats in both

groups stabilized after 8 weeks. This result suggests that HLD has a positive therapeutic effect on the forelimbs of rats with lymphedema and could be combined with VLNF transfer.

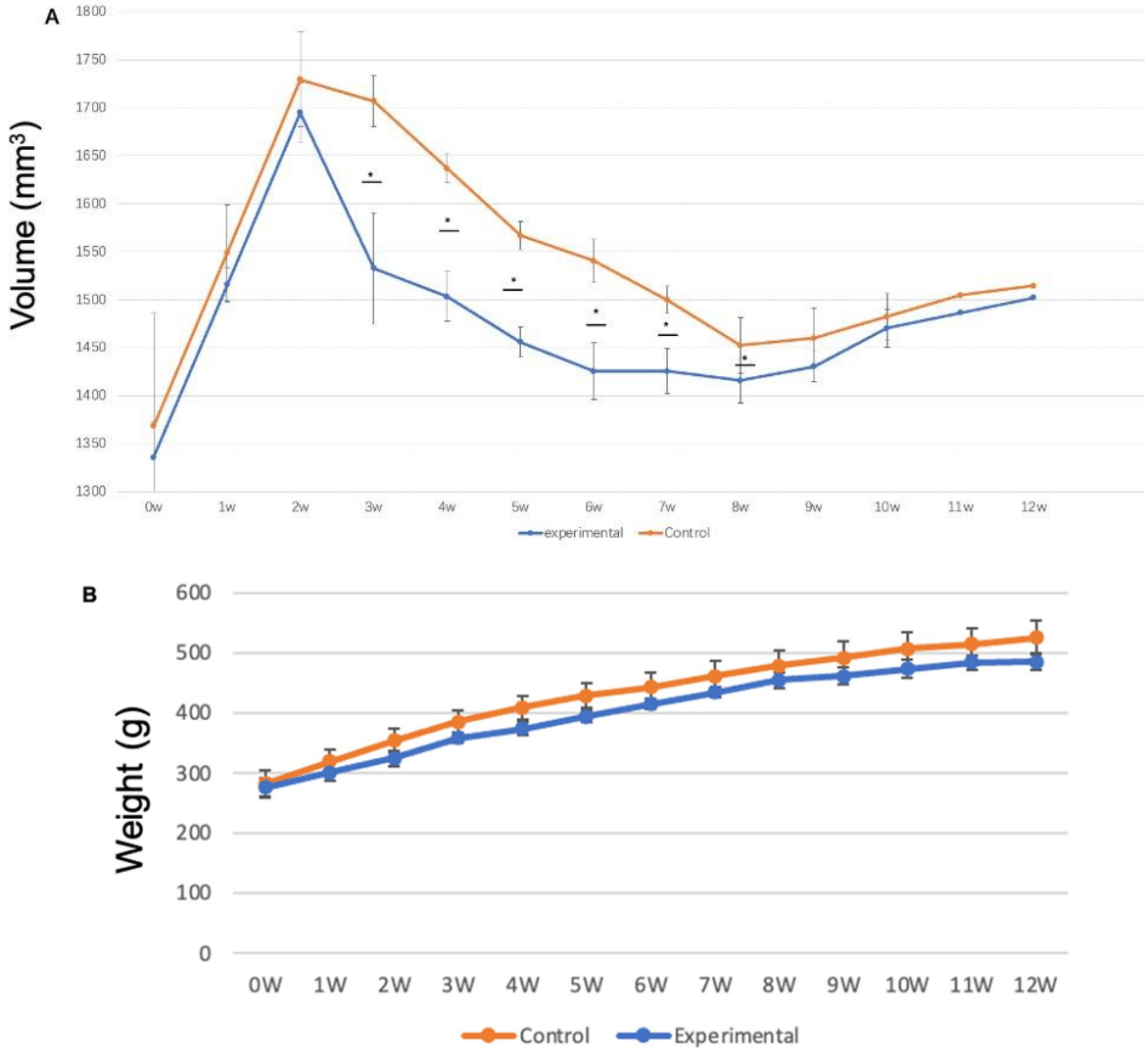


Figure 5. Forelimb volume and Body weight in rats.

(A) Forelimb volume of rats in each group at different time periods. *p<0.05. (B) Body weight changes of the rats in each group during the follow-up.

3.2. Changes in ICG drainage patterns

We collected specific values of the percentage of the various ICG drainage patterns in the rat forelimb each week (Figure. 6A, 6B and 6C). Overall the proportion shows that Linear patterns were more predominant in the experimental group with a more pronounced upward trend. Also, we found that after fourth weeks only the linear and splash modes remained. From the fifth week (Figure. 7A), the percentage of linear patterns in the experimental and control groups was 68.3±7.45% and 56.7±3.58%, respectively, and the difference was statistically significant ($p<0.01$). The Significant p-values were 0.004. While the percentage of sputtering mode was 31.7±7.45% and 43.3±3.58%, respectively, and the difference was statistically significant ($p<0.01$). The Significant p-values were 0.004 (Figure. 7A).

Referring to the above data, we found that both the control and experimental groups showed disturbances in lymphatic drainage patterns four weeks after the start of the experiment. After the fourth week, the lymphatic drainage pattern began to stabilize gradually, but the percentage of linear pattern in the experimental group was higher than that in the control group, and the percentage of splash pattern was lower than that in the control group. In addition, from the eighth week, the proportion of these two drainage patterns in each group tended to stabilize.

A

	1W					7W			
	Linear	splash	stardust	diffuse		Linear	splash	stardust	diffuse
Control	13.3%	33.3%	26.7%	26.7%	Control	60.0%	40.0%	0%	0%
Experimental	14.3%	21.4%	28.6%	35.7%	Experimental	60.0%	40.0%	0%	0%
	2W					8W			
	Linear	splash	stardust	diffuse		Linear	splash	stardust	diffuse
Control	28.6%	35.7%	21.4%	14.3%	Control	60.0%	40.0%	0%	0%
Experimental	55.6%	22.2%	0%	22.2%	Experimental	66.7%	33.3%	0%	0%
	3W					9W			
	Linear	splash	stardust	diffuse		Linear	splash	stardust	diffuse
Control	41.7%	33.3%	8.3%	16.7%	Control	54.5%	45.5%	0%	0%
Experimental	42.9%	28.6%	7.1%	21.4%	Experimental	75.0%	25.0%	0%	0%
	4W					10W			
	Linear	splash	stardust	diffuse		Linear	splash	stardust	diffuse
Control	50.0%	33.3%	16.7%	0%	Control	60.0%	40.0%	0%	0%
Experimental	60.0%	40.0%	0%	0%	Experimental	75.0%	25.0%	0%	0%
	5W					11W			
	Linear	splash	stardust	diffuse		Linear	splash	stardust	diffuse
Control	50.0%	50.0%	0%	0%	Control	54.5%	45.5%	0%	0%
Experimental	60.0%	40.0%	0%	0%	Experimental	75.0%	25.0%	0%	0%
	6W					12W			
	Linear	splash	stardust	diffuse		Linear	splash	stardust	diffuse
Control	60.0%	40.0%	0%	0%	Control	54.5%	45.5%	0%	0%
Experimental	60.0%	40.0%	0%	0%	Experimental	75.0%	25.0%	0%	0%

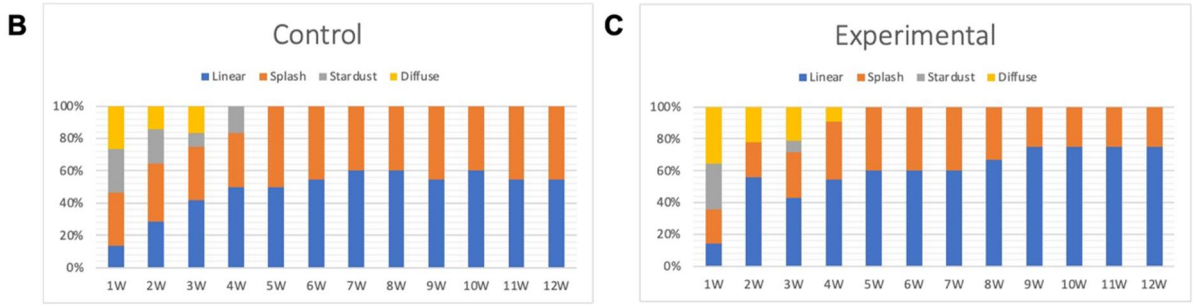


Figure 6. Proportion of ICG drainage patterns.

(A). Specific values for the percentage of various drainage patterns in the control and experimental groups each week. (B) Percentage of various ICG drainage patterns in the control group. (C) Percentage of various ICG drainage patterns in the experimental group.

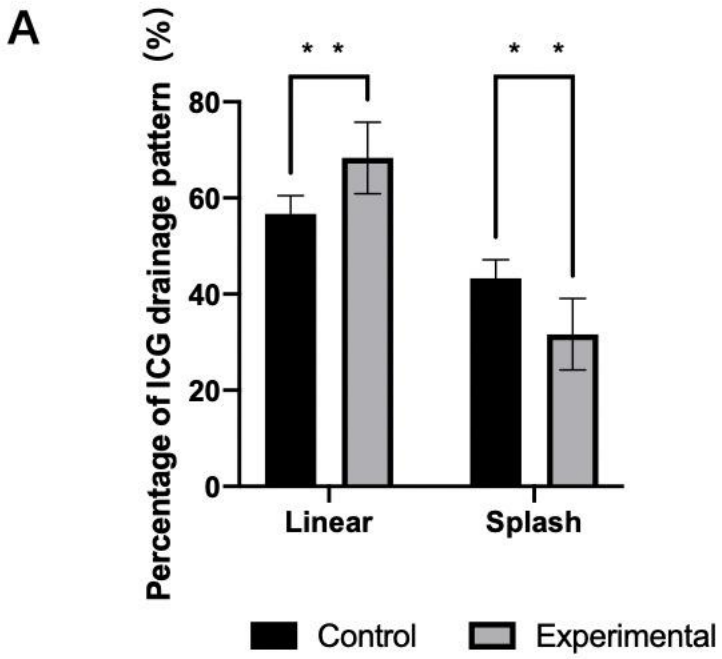


Figure 7. Percentage of ICG drainage pattern.

(A) From the fifth week, the proportion of linear mode and sputtering mode in the control group and the experimental group was compared. **p<0.01.

3.3. Change in Velocity of ICG drainage

By monitoring the intensity of lymphatic drainage, the lymphatic flow was drained into the transferred lymph node after second week in the experimental group, while lymphatic drainage was observed after fourth week in the control group (Figure. 8A). In the comparison of transfer velocity, the velocity in the control group was 0, 0.18±0.01, 0.19±0.01, and 0.25±0.01 in weeks 2, 4, 8, and 12, respectively (Figure. 9A and 9B), while the velocity in the experimental group were 0.09±0.04, 0.16±0.01, 0.25±0.01 and 0.32±0.02 (Figure. 9C and 9D). Under normal conditions, the velocity of drainage of normal axillary lymph nodes in rats is close to 0.32±0.04.

As the treatment progressed, the data from the two groups began to show statistically significant differences at the eighth week ($p<0.05$). The Significant p-values were 0.034 and 0.022, respectively. The results suggest that HLD injection improves drainage outcomes after VLNF transfer. In addition, the experimental group approached the velocity of transfer with normal LNs at the end, which further illustrates the positive effect of HLD injection treatment on VLNF transfer.

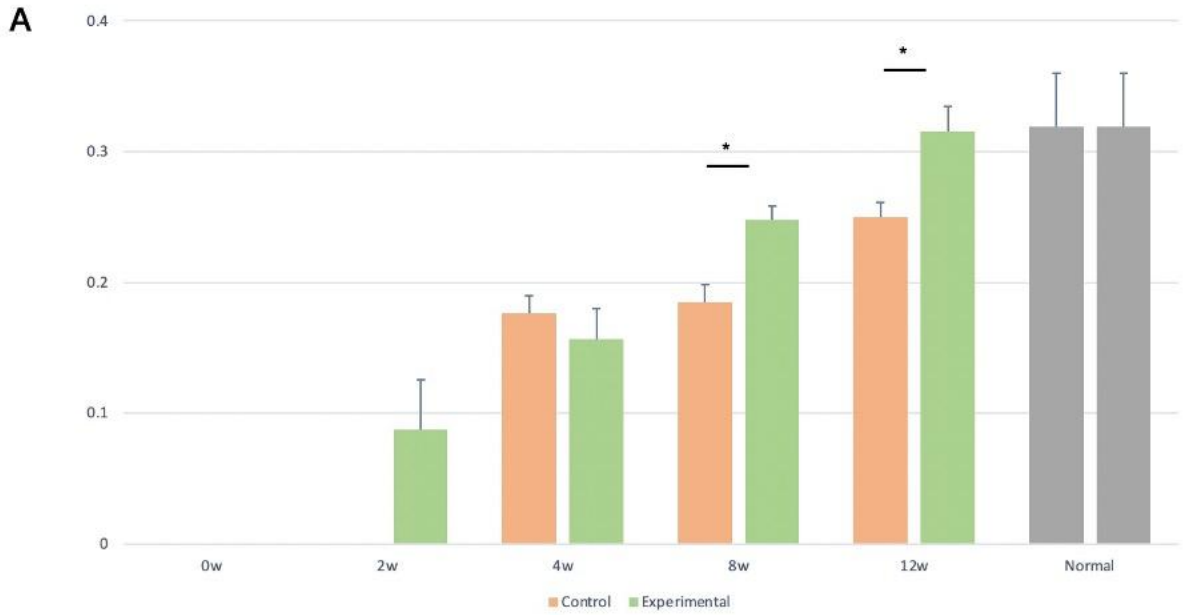


Figure 8. The overall of results of velocity of LN drainage.

(A) Velocity of LN drainage in the experimental and control groups. *p<0.05.

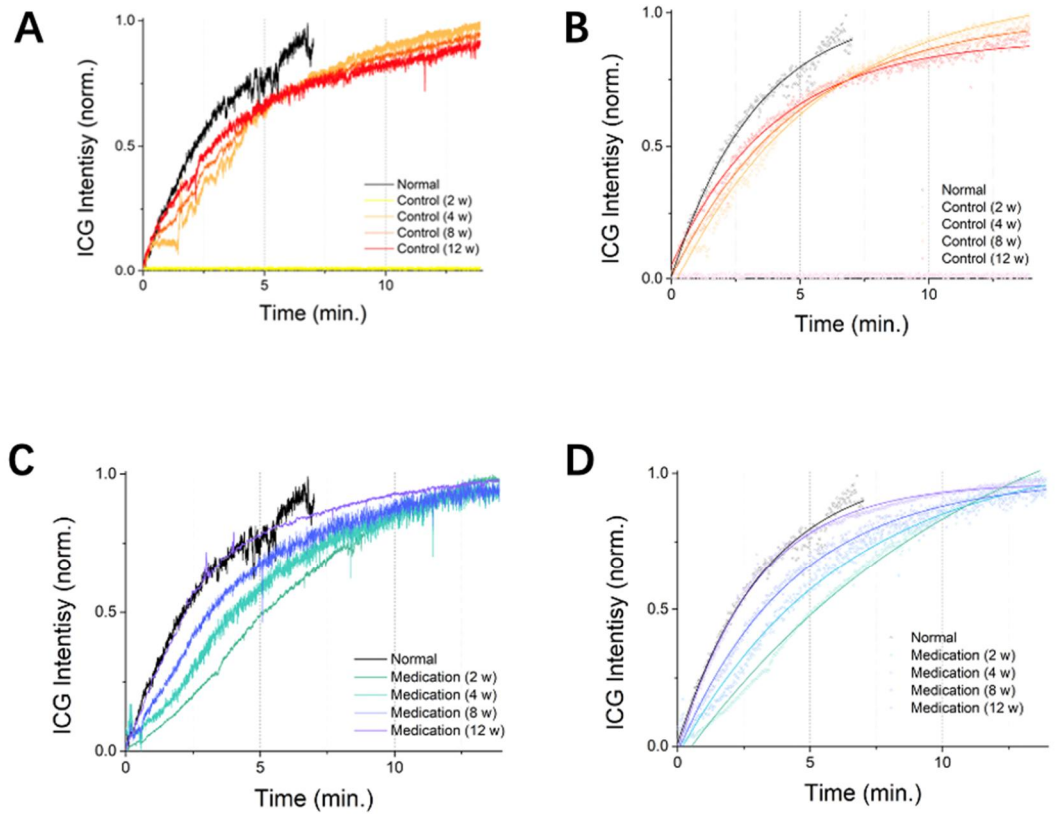


Figure 9. The Velocity of LN drainage in each group.

(A) The velocity of LN transfer in the control group. (B) All data in the control group were standardized by asymptotic regression function. (C) The velocity of LN transfer in the experimental group. (D) All data in the experimental group were standardized by asymptotic regression function.

3.4. Redissect observation

At the fourth week of follow-up (Figure. 10A and 10D), we did not find blue-stained lymphatic tissue under direct visualization, but in the experimental group, we found that the tissue around the lymph node flap was smoother and could be separated by gentle blunt dissection. At the eighth week of follow-up (Figure. 10B and 10E), the control group showed punctiform blue-stained lymphatic tissue and required sharp dissection to reveal the lymph node flap, whereas in the experimental group the blue-stained lymphatic tissue was continuous and the tissue was more easily peeled. At the twelfth week of follow-up (Figure. 10C and 10F), large areas of blue-stained lymphatic tissue were present in the experimental group, and lymphangiogenesis could be identified, which were not present in the control group, but there was extensive scarring around the lymph node flap in the control group, and the redissection took longer than in the experimental group.

This assessment was performed by two independent reviewers in a blinded manner. The overall results of this section showed that the experimental group had less fibrosis than the control group, while in the same period of comparison, it was visualized under the microscope that the experimental group had more regeneration of blue-stained lymphatic vessels than the control group.

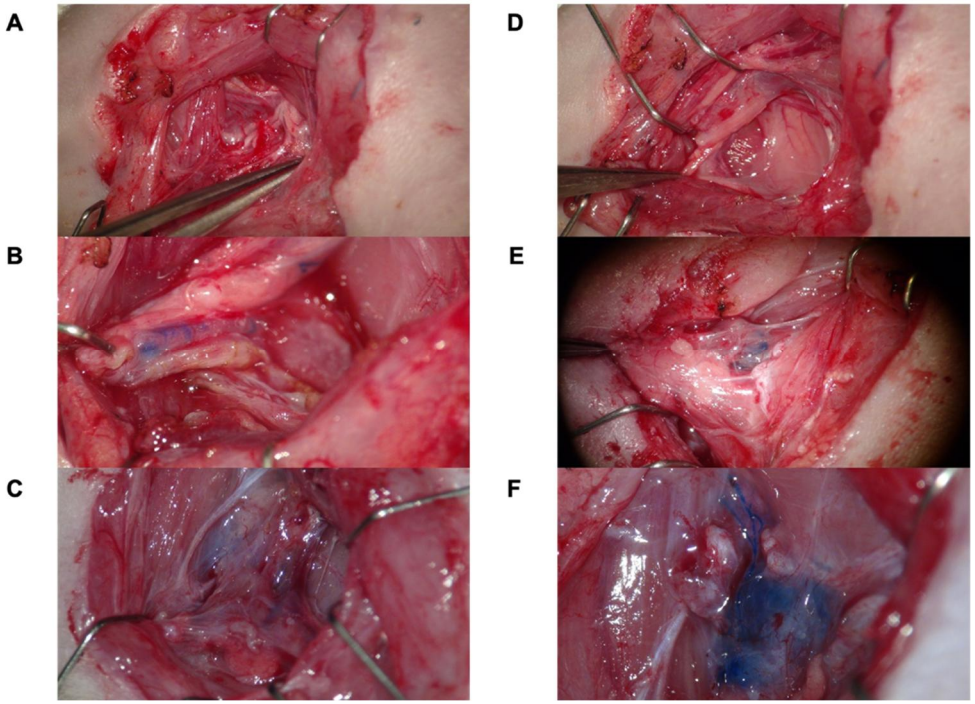


Figure 10. Redissect observation of the condition around the VLNF.

(A) The control group was followed up at the fourth week. (B). The control group was followed up at the eighth week (C) The control group was followed up at the twelfth week. (D) The experimental group was followed up at the fourth week. (E) The experimental group was followed up at the eighth week. (F) The experimental group was followed up at the twelfth week.

3.5. Hyaluronidase promotes lymphangiogenesis in the model

In our experiments, at the first week after the start of the experiment, no normal lymphatic vessels were found around the flap, further indicating that our procedure successfully simulated VLNF transfer. The number of lymphatic vessels in the experimental group was 1.77±0.58, 5.77±0.52, 8.00±1.23, and 10.33±1.53 at weeks 3, 6, 9, and 12, respectively, compared with 1.33±0.58, 3.00±1.23, 5.3±0.30, and 7.3±0.57 in the control group, respectively, with statistically significant differences ($p<0.05$) between the two groups except for the third week (Figure. 11A). The Significant p-values were 0.028, 0.014 and 0.014, respectively.

The number of lymphatic vessels around the transferred VLNF was counted by randomizing the field of view (Figure. 12A and 12B). The above results indicate that after combined HLD injection treatment, an increased number of lymphatic vessels around the flap was demonstrated, further indicating that HLD promotes lymphangiogenesis.

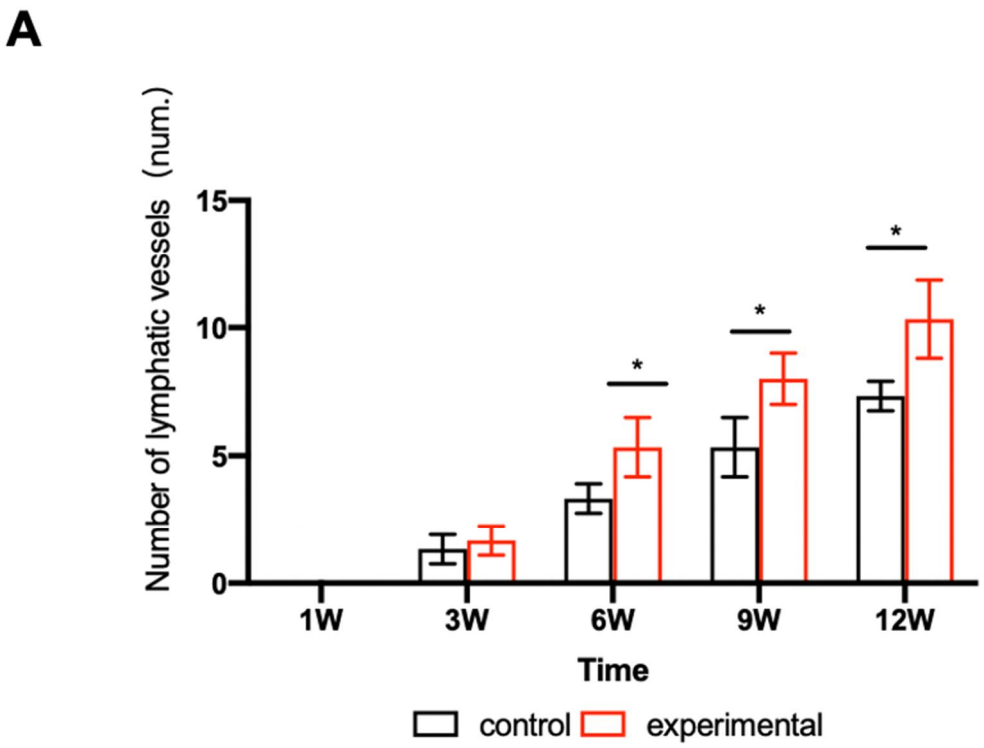


Figure 11. Lymphatic vessel count.

(A) Lymphatic vessels were searched and counted around the VLNF under random visual field. *p<0.05.

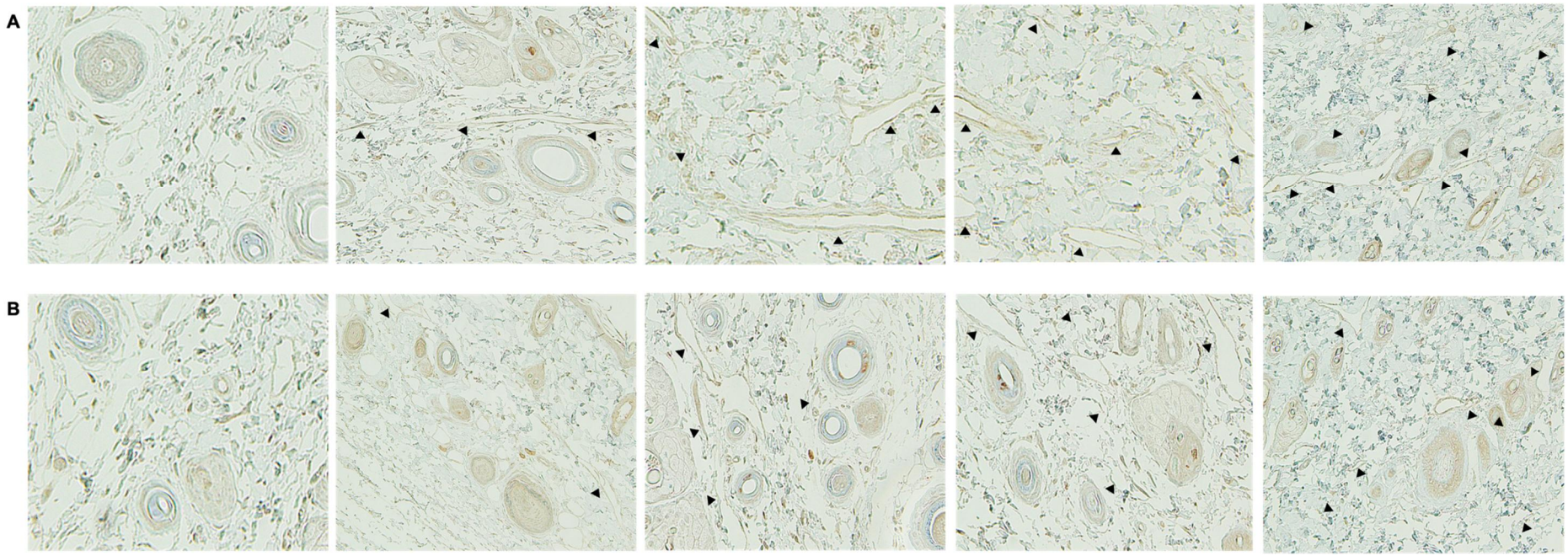


Figure 12. Lymphatic vessels assessment with LYVE-1 staining.

(A) From left to right are representative pathological photographs of the control group at week 1, 3, 6, 9 and 12 respectively. (B) From left to right are representative pathological photographs of the experimental group at week 1, 3, 6, 9 and 12 respectively. The black arrow shows the location of the lymphatic vessels.

3.6. Hyaluronidase inhibits fibrosis in the model

In the experimental group, the average fiber area proportion of randomly selected images after dyeing was 38.93±7.19%, 43.50±1.18%, 41.21±0.91%, 32.78±2.39%, 21.58±2.28% at 1, 3, 6, 9, and 12 weeks, respectively. The control group was 40.10±7.04%, 54.79±2.49%, 51.67±4.69%, 44.38±1.33%, 35.09±1.38%, respectively (Figure. 13A). The data showed that the average fibrosis area of the experimental group was less than that of the control group at all periods, and the difference was statistically significant except for the first week ($p<0.05$). The Significant p-values were 0.035, 0.026, 0.030 and 0.012, respectively.

The level of fibrosis around the VLNF was shown by Masson's Trichrome staining and collagen fibers were shown in blue, in the typical pathological pictures randomly selected, the blue area showed a downward trend with the progress of treatment (Figures. 14A and 14B). We can conclude that HLD combination therapy can reduce the fibrosis level around the VLNF.

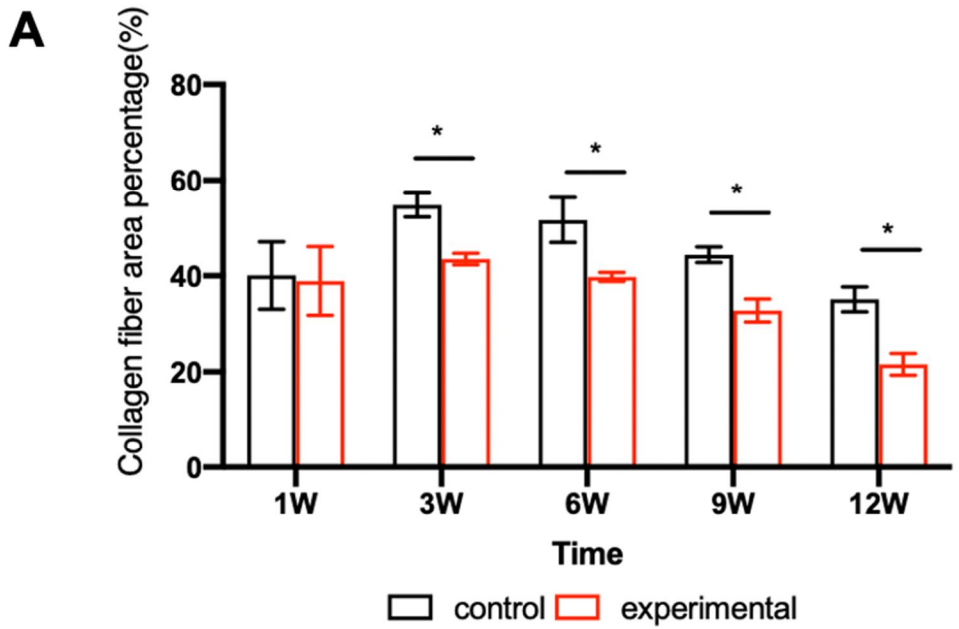


Figure 13. Proportion of fibrosis area.

(A) The degree of fibrosis was compared by calculating the area proportion of collagen fiber staining at different time periods. *$p<0.05$.

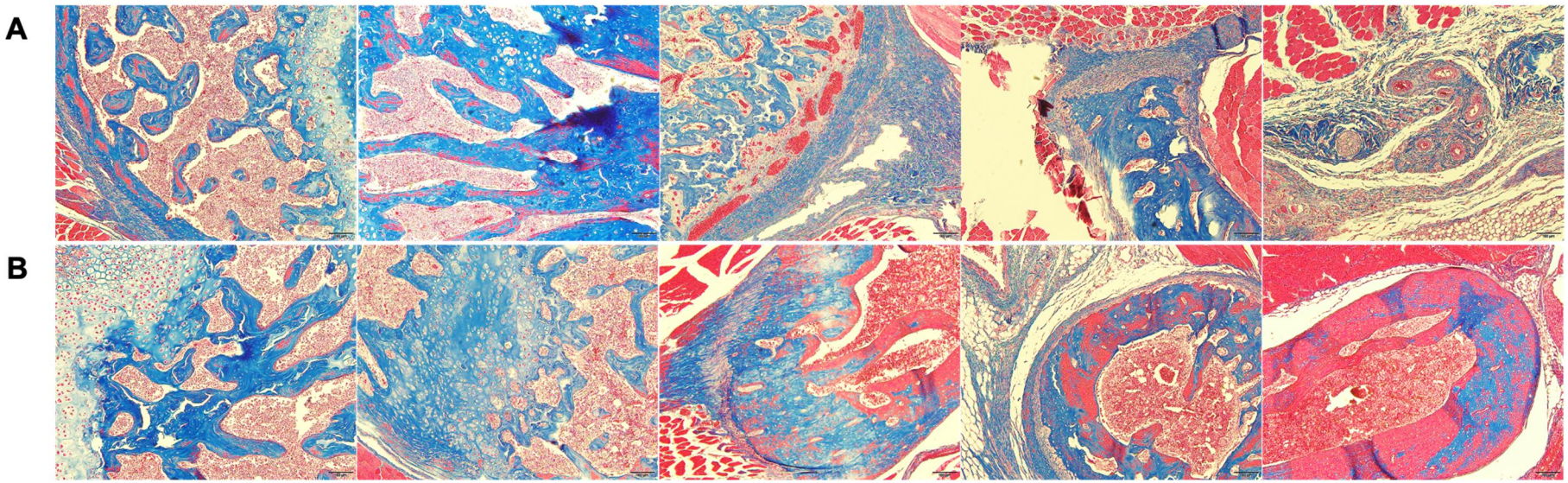


Figure 14. Fibrosis assessment with Masson's trichrome staining.

(A) From left to right are representative pathological photographs of the control group at weeks 1, 3, 6, 9, and 12 respectively. (B) From left to right are representative pathological photographs of the experimental group at weeks 1, 3, 6, 9, and 12 respectively.

4. DISSCUSSION

lymphedema is a debilitating and physiological disease, associated with functional deficits in the affected limb, decreased quality of life, and impaired aesthetics, as well as increased treatment costs [27]. Various strategies have been described for the treatment of lymphedema, including surgical, medical, and rehabilitation therapies [3]. As aforementioned, VLNF transfer is undoubtedly one of the first lines of treatment for lymphedema of all etiologies, especially moderate to severe lymphedema based on ISL staging, and could reverse the pathophysiological process of lymphedema [28]. Despite VLNF transfer having made great progress in the treatment of lymphedema, there is still a major issue that poor function of the transferred lymph node flap due to scar adhesions and tissue fibrosis proliferation in clinical applications. Likewise, an increase in tissue fibrosis could influence the spontaneous anastomosis of lymphatic vessels leading to reduced lymphangiogenesis [12].

Hyaluronidase (HLD) is a family of natural enzymes. They are used in a wide range of medical fields including Skin infiltration, skin graft harvesting, liposuction analgesia, dermal filler problems, treating extravasation injuries, preventing and controlling edema, treating ganglia, and scar management [29]. The underlying mechanisms lie in HLD breaking down hyaluronic acid by cleaving glycosidic bonds and other acid mucopolysaccharides in the connective tissue. The hyaluronic acid oligosaccharides formed after decomposition affect the production of fibroblasts, smooth muscle cells, and vascular endothelial cells. In addition, hyaluronidase reduces the production of inflammatory mediators, IL-8, LTB4, and LTC4 [30].

Recent studies have shown that hyaluronic acid expression increases in tissues with lymphedema and HLD enhanced lymphangiogenesis, induced the proliferation of lymphatic endothelial cells, and reduced extracellular matrix proliferation in lymphedematous limbs [31].

In our study, we established an animal model of stable VLNF transfer for lymphedema and set injection of HLD as the sole variable. It is imperative to note that this animal model has proven to be reliable in our previous experiments. Other than that, in the current experiment, we found obvious swelling of the forelimbs of the rats at the early stage of HLD treatment, and the disturbance of the lymphatic drainage pattern and the disappearance of the drainage pathways were further evidence of the validity of this model. In addition, for the dose of HLD injected in rats, we consulted other research literature and found that a single daily dose of 1500 units can be used as an effective dose for treatment in rats [30].

In the comparison of treatment results, the experimental group, namely the HLD injection group, showed more positive treatment results, specifically, an accelerated decrease in forelimb swelling volume in rats, a higher percentage of linear patterns in the ICG drainage pattern, earlier recovery of lymphatic pathways, and a drainage velocity closer to that of normal LNs. what's more, we must focus on three-time points: the second week, the fourth week, and the eighth week. In the rat experiment, there was a statistical difference in the volume of the limbs between the two groups, while the rats in the experimental group began to resume normal drainage of the lymph nodes in terms of the velocity of drainage. And from the fourth week onwards, the lymphatic drainage pattern began to transition from disorder to stability, with only linear and splash patterns remaining, and the difference between the two patterns appeared

statistically significant. At the same time, the rats in the control group began to resume normal lymph node drainage at the velocity of drainage, and the difference in forelimb volume between the two groups increased. After the eighth week, most of the results began to stabilize, including forelimb volume, lymphatic drainage pattern, and lymph node metastasis rate. Although the differences between the groups were not statistically significant, the experimental groups were all superior to the control group. Therefore, we assumed that these three-time points may be related to lymphatic vessel regeneration and lymphatic vessel proliferation around the VLNF. It was shown that lymph nodes have a high capacity for spontaneous regeneration, with lymphangiogenesis occurring rapidly after lymphadenectomy, taking approximately 3 weeks in rodents [32-33].

On the other hand, based on our results, HLD positively influences the lymphatic vessel regeneration in the transferred VLNF and also lowers the degree of fibrosis around the VLNF transfer. First, in the redissect observation, the blue-stained lymphoid tissues were more obvious in the experimental group as the treatment progressed, while the inter-tissue peeling was easier. In particular, at eight weeks, lymphatic regeneration was evident, and linear lymphatics appeared around the lymph node flap. Second, strong evidence in histological analysis suggested that HLD injection significantly increased the expression of lymphatic vessels in LYVE-1 staining from the third week onwards, while MT staining showed that HLD decreased collagen fiber deposition and synthesis compared with the control group from the third week. This further indicates that HLD can stimulate lymph endothelial cell regeneration and inhibit extracellular matrix fibrosis, which may be related to the products of HLD

decomposition [24]. Likewise, This part of the results further demonstrates that HLD promotes lymphatic vessel regeneration, which generally begins at 2-3 weeks, and reduces the level of fibrosis around the flap, raising the threshold for the number of lymphatic vessels regenerated, which could explain the continued superiority of the experimental group over the control group at the end of the treatment period.

There were several limitations to our study. First, this is an animal experimental study, and the experimental data obtained can only be used as a reference for preclinical treatment, including the injection dose of HLD. Furthermore, to reduce the difficulty of the experimental operation and increase the success rate of surgery, we made an animal model simulating VLNF transfer for lymphedema instead of completely free VLNF transplantation. Although this model showed similar treatment outcomes, the pathophysiological progression remained different. Second, Volume measurement error is inevitable, in addition, for descriptive results, only through my subjective observation, which may lead to the deviation of the results.

In summary, this is an innovative finding that, in animal studies, combined HLD injection helps to optimize the efficacy of VLNT transfer for lymphedema, including accelerated reduction of limb swelling and early restoration of normal lymphatic drainage pattern, which is associated with reduced fibrosis around VLNFs and lymphangiogenesis. Nevertheless, further, exploration remains if it is to be applied to the clinic.

5. REFERENCE

1. Schaverien MV, Coroneos CJ. Surgical treatment of lymphedema[J]. *Plast Reconstr Surg*, 2019, 144(3): 738-758.

2. Kung TA, Champaneria MC, Maki JH, et al. Current concepts in the surgical management of lymphedema[J]. *Plast Reconstr Surg*, 2017, 139(4): 1003e-1013e.

3. Chang DW, Masia J, Garza R 3rd, et al. Lymphedema: surgical and medical therapy[J]. *Plast Reconstr Surg*, 2016, 138(3 Suppl): 209S-218S.

4. Greene AK, et al. Lymphedema: presentation, diagnosis, and treatment[M]. *Springer International Publishing Switzerland*, 2015: 59-60.

5. Connell FC, Gordon K, Brice G, et al. The classification and diagnostic algorithm for primary lymphatic dysplasia: An update from 2010 to include molecular findings[J]. *Clin Genet,* 2013, 84(4): 303–314.

6. Ciudad P, Sabbagh MD, Agko M, et al. Surgical management of lower extremity lymphedema: a comprehensive review[J]. *Indian J Plast Surg*, 2019, 52(1): 81-92.

7. Rosian K, Stanak M. Efficacy and safety assessment of lymphovenous anastomosis in patients with primary and secondary lymphedema: A systematic review of prospective evidence[J]. *Microsurgery*, 2019, 39(8): 763-772.

8. Dayan JH, Ly CL, Kataru RP, et al. Lymphedema: pathogenesis and novel therapies[J]. *Annu Rev Med*, 2018, 69: 263-276.

9. Jiang X, Nicolls MR, Tian W, et al. Lymphatic dysfunction, leukotrienes, and lymphedema[J]. *Annu Rev Physiol*, 2018, 80: 49-70.

10. Buragadda S, Alhusaini AA, Melam GR, et al. Effect of complete decongestive therapy and a home program for patients with post mastectomy lymphedema[J]. *J Phys Ther Sci*, 2015, 27(9): 2743-2748.

11. Gallagher K, Marulanda K, Gray S. Surgical Intervention for Lymphedema[J]. *Surg Oncol Clin N Am*, 2018, 27(1): 195-215.

12. Granzow JW. Lymphedema surgery: the current state of the art[J]. *Clin Exp Metastasis*, 2018, 35(5-6): 553-558.

13. Allen RJ Jr, Cheng MH. Lymphedema surgery: patient selection and an overview of surgical techniques[J]. *J Surg Oncol,* 2016, 113(8): 923-931.

14. Tourani SS, Taylor GI, Ashton MW. Vascularized lymph node transfer: a review of the current evidence[J]. *Plast Reconstr Surg*, 2016, 137(3): 985-993.

15. Pappalardo M, Patel K, Cheng, MH. Vascularized lymph node transfer for treatment of extremity lymphedema: An overview of current controversies regarding donor sites, recipient sites and outcomes[J]. *J Surg Oncol*, 2018, 117(7): 1420-1431.

16. Hanson SE, Chang EI, Schaverien MV, et al. Controversies in surgical management of lymphedema[J]. *Plast Reconstr Surg Glob Open*, 2020, 8(3): e2671.

17. Greene AK, et al. Lymphedema: presentation, diagnosis, and treatment[M]. *Springer International Publishing Switzerland*, 2015: 269-270.

18. Ito R, Zelken J, Yang CU, et al. Proposed pathway and mechanism of vascularized lymph node flaps[J]. *Gynecol Oncol*, 2016, 141(1): 182-188.

19. Asaad M, Hanson SE. Tissue engineering strategies for cancer-related lymphedema[J].*Tissue Eng Part A,* 2021, 27(7-8): 489-499.

20. Alderfer L, Wei A, Hanjaya-Putra D. Lymphatic tissue engineering and regeneration[J]. *J Biol Eng,* 2018, 17(12): 32.

21. Mishra VK, Shih HH, Parveen F, et al. Identifying the therapeutic significance of mesenchymal stem cells[J]. *Cells,* 2020, 9(5): 1145.

22. Hu LR, Pan J. Adipose-derived stem cell therapy shows promising results for secondary lymphedema[J]. *World J Stem Cells,* 2020, 12(7): 612-620.

23. Jung H. Hyaluronidase: an overview of its properties, applications, and side effects[J]. *Arch Plast Surg,* 2020, 47(4): 297-300.

24. Roh K, Cho S, Park JH, et al. Therapeutic effects of hyaluronidase on acquired lymphedema using a newly developed mouse limb model[J]. *Exp Biol Med (Maywood)*, 2017, 242(6): 584-592.

25. Hartiala P, Saaristo AM. Growth Factor Therapy and Autologous Lymph Node Transfer in Lymphedema[J]. *Trends Cardiovasc Med*, 2010, 20(8): 249-253.

26. Hartiala P, Suominen S, Suominen E, et al. Phase 1 Lymfactin® Study: Short-term Safety of Combined Adenoviral VEGF-C and Lymph Node Transfer Treatment for Upper Extremity Lymphedema[J]. *J Plast Reconstr Aesthet Surg*, 2020, 73(9): 1612-1621.

27. Spörlein A, Will PA, Kilian K, et al. Lymphatic tissue engineering: a further step for successful lymphedema treatment[J]. *J Reconstr Microsurg*, 2021, 37(6): 465-474.

28. Ramachandran S, Chew KY, Tan BK, et al. Current operative management and therapeutic algorithm of lymphedema in the lower extremities[J]. *Asian J Surg*, 2021, 44(1): 46-53.

29. Sharma DSC, Lahiri MA. Use of hyaluronidase in plastic surgery: A review[J]. *J Plast Reconstr Aesthet Surg*, 2021, 74(7): 1610-1614.

30. Kim SK, Yang JY, Kim CW, et al. The Effect of hyaluronidase on the fat graft[J]. *J Craniofac Surg*, 2020, 31(3): 618-621.

31. Cho S, Roh K, Park J, et al. Hydrolysis of hyaluronic acid in lymphedematous tissue alleviates fibrogenesis via T(H)1 cell-mediated cytokine expression[J]. *Sci Rep*, 2017, 24:7(1): 35.

32. Saaristo AM, Niemi TS, Viitanen TP, et al. Microvascular breast reconstruction and lymph node transfer for postmastectomy lymphedema patients[J]. *Ann Surg*, 2012, 255(3): 468-473.

33. Ishikawa K, Maeda T, Funayama E, et al. Feasibility of pedicled vascularized inguinal lymph node transfer in a mouse model: a preliminary study[J]. *Microsurgery,* 2019, 39(3): 247-254.

6. ABSTRACT IN KOREAN

배경 및 목적: 림프부종은 전 세계적으로 최대 2 억 5 천만 명에게 영향을 미치는 압도된 림프계의 만성, 쇠약화 및 소모성 상태이다. 현재 림프부종에 대한 치료법은 아직 없지만 외과적 치료와 약물 치료에서 약간의 돌파구를 마련하고 있다. 림프부종에 대한 생리학적 외과적 치료, 특히 혈관화된 림프절 피판 (VLNF) 전이술은 림프부종의 임상적 효과를 개선하고 환부의 기능적 회복을 촉진할 뿐만 아니라 림프부종의 병리학적 과정을 역전시켜 봉와직염의 발병률을 감소시키는 것이 입증되었다. 그러나, 전이 플랩은 주변 조직과의 섬유성 유착 및 세포외 기질 침착을 형성하여 림프관 신생을 차단하고 림프액 폐색을 유발할 수 있으며, 이는 VLNF 전이 효과에 영향을 미치는 가장 큰 문제이다. 또한, 히알루로니다아제는 림프부종 사지의 림프관 신생을 향상시킬 뿐만 아니라 조직 섬유증의 수준을 감소시킨다. 본 연구의 목적은 히알루로니다아제 주입과 결합된 VLNF 전이의 효과를 평가하는 것이다.

재료 및 방법: 체중 250-300 g 의 수컷 성체 Sprague-Dawley(SD) 쥐 34 마리를 실험에 사용하였다. 첫째, 모든 쥐는 림프부종에 대한 VLNF 전이 모델로 설정하였다. 그 후 히알루로니다아제의 주입 여부에 따라 실험군과 대조군의 2 개 군으로 분류하였다. 각 그룹은 세 부분으로 나뉜다. 파트 I 에서 6 마리의 쥐에 대하여 매주 포스트 소프트웨어 분석을 사용하여 상지 부피를 기록하고 NIR 인도시아닌 그린 이미징에서 림프 배액 패턴을 관찰하였다. 파트 II 에서는 4 주마다 1 마리의 쥐를 희생시켜 수술 부위를 재해부하여 현미경으로 림프관 형성 단계와 주변 섬유화 정도를 관찰하였다. 또한, 이 그룹의 쥐에서 2 주마다 NIR 인도시아닌 그린 이미징

시스템으로 림프 수송 속도를 계산하였다. 파트 III 에서는 림프관 신생 및 상지 섬유증을 정량화하기 위한 병리학적 검사를 위해 3 주마다 2 마리의 쥐를 희생시켰다. 실험 12 주 후, 모든 쥐를 안락사시켰다.

결과: 실험군에서 대조군보다 상지 부피의 변화가 더 컸다. 또한 실험이 진행됨에 따라 ICG 에 의해 표시되는 선형 배액 패턴이 실험군에서 더 높은 비율을 차지하였고 상승 경향이 더욱 뚜렷하였다. 유사하게 림프절 전이 속도의 비교에서도 실험군의 데이터가 대조군보다 속도가 매우 빨랐고 치료 후기의 정상 림프절 전이 속도에 거의 근접하였다. 더욱이, 병리학적 결과는 실험군에서 림프관의 함량이 더 많은 반면 콜라겐 섬유의 함량은 더 적은 것으로 나타났다.

결론: 동물 실험에서 히알루로니다아제 주입은 림프부종에서 VLNF 전이술의 치료 효과를 최적화할 수 있다. 이는 복합 치료가 림프관 신생을 촉진하고 VLNF 주변의 섬유화를 감소시키기 때문이다. 이러한 효과에도 불구하고, 이에 대한 임상 적용은 더 많은 연구가 필요하다.

키워드: 혈관화된 림프절 피판; 히알루로니다아제; 림프부종; 쥐; 치료