

The Study of Animal Model of Lymphedema Using the Mouse Tail

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Objectives: To investigate the time course of the development of acquired and experimental lymphedema.

Methods: We studied an experimental model of acute post - surgical lymphedema in the tails of female hairless mice. The procedures that remove the skin and subcutaneous tissue in tails of the mice (5-10 mm from tail base) were performed, and then the murine has acquired lymphatic insufficiency. We measured volume of the tails in 2 times per week for 5 weeks, histological biopsy, and lymphoscintigraphy to assess lymphatic flow.

Results: There was gradually increased volume of the tails and observed twice volume at post - surgical 18 days. In lymphoscintigraphy, we identified decreased lymphatic flow and dermal back flow in the tails. Histological biopsy showed inflammatory response that was edema and increased neutrophils in epidermis and subdermis, and lymphatic microvascular dilatation.

Conclusions: We have a mouse model of acute acquired lymphedema. This post - surgical murine tail model of lymphedema can be used to simulate an attribute of human lymphedema and provides knowledge about functional and structural alterations of lymphedema.

Key Words: Animal model, Lymphedema, Pathologic findings

In most cases, lymphedema is generated indirectly due to various causes, such as malignant tumor, infection, injury, and filariasis. In particular, a large number of cases are related with malignant tumor or the treatment of malignant tumor. The most common cause of lymphedema in the upper limb is related to breast cancer. The reported incidence of breast cancer related lymphedema has ranged from 2.4% to 49%.^{1,2} In addition to edema, lymphedema patients complain of discomfort related with pain, tingling, or heaviness.³ The important aspects of lymphedema treatment are the functional decline of edematous parts, cosmetic aspects according to change of appearance, and prevention of infection.

As the lymphatic undercurrent is rather complex, structural and functional knowledge about lymphatic vessels must be accompanied to understand lymphedema. Although lymphedema has recently been reported to be related with inflammatory reaction in the tissue, it is also understood as a direct or indirect result of the damaged immune system.⁴ And whereas mechanism of lymphedema has been previously reported as Stopcock to begin from hand or foot edema due to declined lymph flow of the axilla or groin. However, some researches have been conducted about regional distribution, recently. And regional filtration rate has been raised as the mechanism of lymphedema.

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In this regard, perfect understanding of lymphedema is not yet to be established and experimental research must be continuously progressed to understand the mechanism of lymphedema. However, it is difficult to find the histological changes in lymphedema and to apply various treatment methods in actual patients. Therefore the development of an appropriate animal model will widen general understanding about the mechanism of lymphedema, and help to develop the treatment of lymphedema. Although research on lymphedema is already being actively conducted using mouse and rabbit models abroad, studies using animal models remain insignificant in this country. This study attempts to present the production method of a secondary lymphedema animal model that is relatively simple and reproducible when compared with previous models.

SUBJECT AND METHOD

1. Research subject

A total of 18 5-week-old ICR female mice (weight: 17–22 g) were classified into experimental group and control group randomly. 12 mice were randomly selected for the experimental group. Ether was used for anesthesia and skin and subcutaneous tissue between from the base 5–10 mm to the distal region

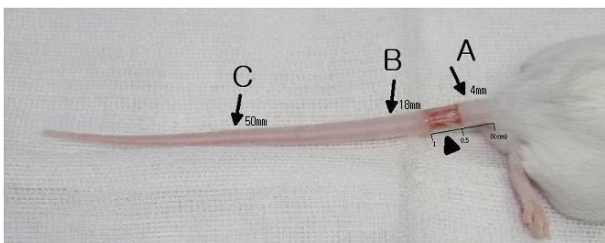


Fig. 1. These photos show parts of surgical resection (arrow head) and histological biopsy (arrow A, B and C)

of the tail were removed (Fig. 1). the mice were isolated from another to prevent damage caused by other mice. Continuous observation was made for 5 weeks. During this period, drugs that may affect research were not administered. Also, 6 female mice were selected as the control group. All mice included in the experiment group and control group were bred in an animal laboratory of 20–25°C, and equal amount of food and water was sufficient provided to both groups.

This study was conducted after receiving the approval of the IACUC (Institutional Animal Care and Use Committee) of Kosin University Gospel Hospital.

2. Research method

1) Volume measurement

At post – surgery 5th week, 4 mice were randomly selected at a 3 and 4 day interval (2 times a week) to measure the tail diameter of 8 parts in 10 mm interval from the base to the distal region of the tail by one physician. Along with a ruler for measuring the length in the measurement process, photos were taken to measure the diameter of each region (Fig. 2). Also, after converting the diameter to circumference, the formula ($V = h (C_1^2 + C_1C_2 + C_2^2)/12\pi$) was



Fig. 2. The diameter of mouse tail was measured with rod ruler by photograph.

used to convert to volume by regarding each segment as a sliced cone.⁶ Four mice were also randomly selected for the control group in the same method to check changes in the volume for 5 weeks after the start of the experiment.

2) Nuclear test

Two weeks after the surgery, 2 mice were randomly selected from both the experimental group and control group to perform lymphoscintigraphy. 100 uCi/0.02 ml ^{99m}Tc – phytate was injected in the skin from the base to 70 mm point of the tail. Ether inhalation anesthesia was administered to prevent movement of mice. Isotope was injected to film images after 10 minutes, 30 minutes, and 1 hour through the low energy general purpose collimator using micro SPECT INFINIA gamma camera (GE medical system, Waukesha, USA) to compare the two groups.

3) Biopsy and immunohistochemistry

From the 1st week to the 5th week after surgical procedure, 2 mice were randomly selected according to the number of week. Anesthesia was administered by using carbon monoxide and the base part of the tail was surgically removed and fixed in formalin. Afterward, 1 mm tissue was collected from the area 4 mm (Fig. 1A), 18 mm (Fig. 1B) and 50 mm from the base part of the tail (Fig. 1C) for biopsy.

One mouse was randomly selected from the contrast group to administer anesthesia by using carbon monoxide. Tissue was collected and fixed in formalin. Identical parts with the experiment group were obtained biopsy. Afterward, paraffin block was produced from the tissue of the experimental group and control group to perform hematoxylin eosin stain and check histological changes.

Furthermore, paraffin block of each subject group was cut into 4µm to undergo the deparaffin process

and formula processing and use Bond Automated Immunohistochemistry (Vision Biosystem LTD, Mount Waverly, Australia) and Bond Polymer Intense Detections System (Vision Biosystem LTD, Mount Waverly, Australia) to perform immunohistochemistry. LYVE – 1 (lymphatic vessel hyaluronan receptor – 1, polyclonal, 1:100, Millipore) was used for the primary antibody. For the negative control group, immunohistochemistry was performed without using primary antibody. Brown changes in the protoplasm of the vascular endothelial cells were judged as positive.

4) Statistical analysis

SPSS for windows (version 14.0) was used for the statistical analysis of mouse tail diameter and volume changes measured in two groups to calculate the mean and standard deviation of the two groups. Wilcoxon rank sum test was used to compare the degree of volume changes between the two groups. Repeat measure ANOVA was used to compare the difference between the proximal and distal parts of edema. Results with p value below 0.05 were analyzed to hold statistical significance.

RESULTS

1. Change in volume

According to the diameter and volume of mouse's tail significant increase was presented in the experiment in comparison with the control group. Significant difference was presented in the volume of the experiment group and control group from day 7 after tissue incision ($P < 0.05$), and this difference was continued after week 5, the end point of experiment. When observing volume changes according to the passing of time, the most distinct

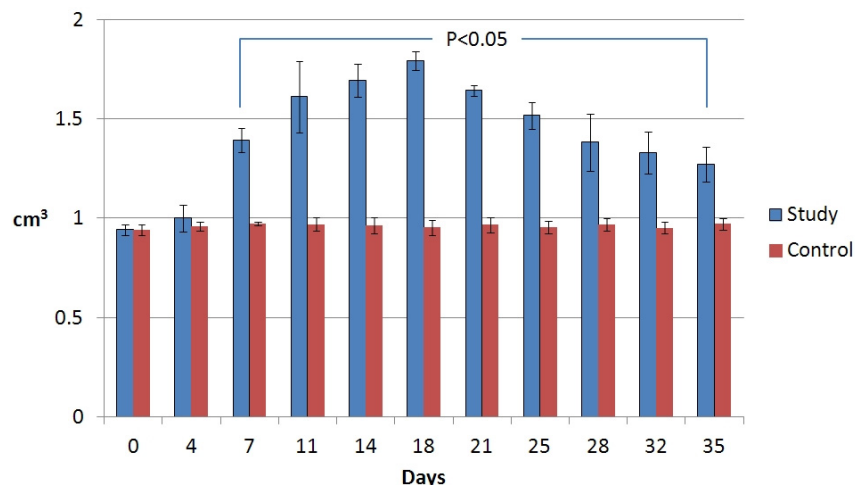


Fig. 3. Tail volume changes following removal of skin and subcutaneous tissues. The study group displayed nearly twice volume at post - 18 days.

Table 1. Change of Diameter on the Mouse Tail

	20 mm from base	50 mm from base
Pre - procedure (mm)	$4.55 \pm 0.19^{*†}$	$3.00 \pm 0.10^{*†}$
After 1 week	$6.25 \pm 0.21^{*†}$	$4.20 \pm 0.24^{*†}$
After 2 week	$7.20 \pm 0.26^{*†}$	$5.40 \pm 0.39^{*†}$
After 3 week	$7.10 \pm 0.12^{*†}$	$5.00 \pm 0.10^{*†}$
After 4 week	$6.18 \pm 0.68^{*†}$	$4.33 \pm 0.46^{*†}$
After 5 week	$5.85 \pm 0.24^{*†}$	$4.23 \pm 0.39^{*†}$

increase was presented in day 18 after surgery with approximately twice larger volume than the control group. Continuous decrease in volume was presented after day 18 (Fig. 3). Furthermore, according to the comparison of the diameter increase in the proximal and distal part of the edema, the proximal part of the edema presented a larger increase than the distal part (Table 1).

2. Nuclear test

Lymphoscintigraphy performed with the two mice randomly selected in the both groups 2 weeks after surgery. The experiment group presented a noticeably reduced lymph flow than the control group to decrease lymph discharge and renal node was presented 30 minutes after the isotope injection. Also, dermal back

flow was observed in the parts of removed skin and tissue under skin to verify lymphedema in the entire mouse tail (Fig. 4).

3. Biopsy and immunohistochemistry

Significant changes were presented according to the passing of time in the tissue fragments gained by randomly selecting 2 mice according to the number of weeks in the experiment group. Changes in the tissue were observed 1 week after the surgery, with especially distinct changes presented in the distal part of the surgical region. The degree of changes was decreased in areas near the distal part of the tail. Although the proximal region of surgery procedure (A) did not present any special changes after the surgical procedure, local edema was presented in the

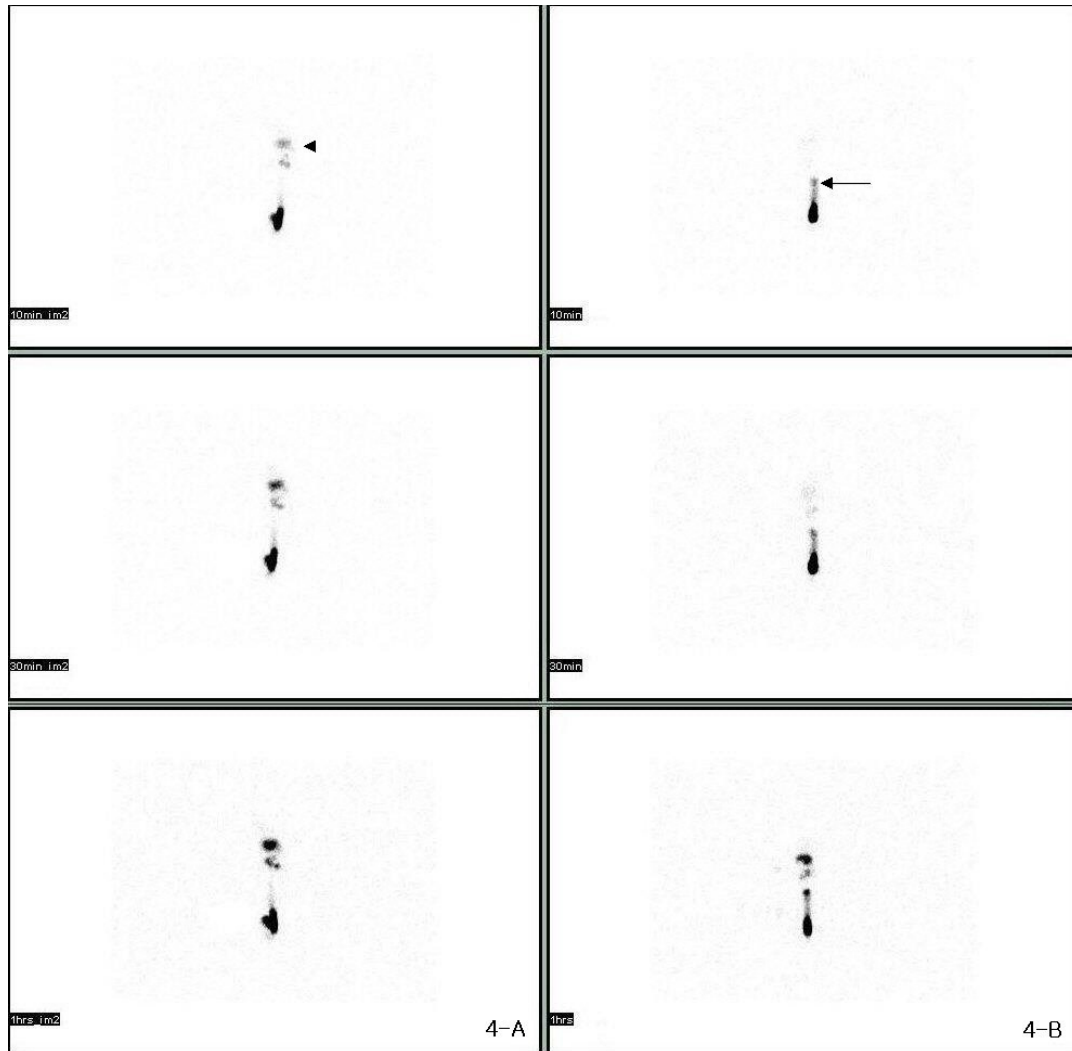


Fig. 4. Lymphoscintigraphy was performed after the intradermal injection of 100 uCi/0.02 ml of filtered ^{99m}Tc - phytate. Dynamic and static images were acquired using a low energy general purpose collimator in a microSPECT INFINIA gamma camera. The renal nodes (arrow head) were visualized significantly in control group (A). But, in study group (B), renal nodes were visualized 30 minute after injection and dermal back flow (arrow) was observed.

tail base 18 mm distal part (B) after 1 week. Overall edema was presented after 2 weeks to be continued to the end of the experiment in week 5. Furthermore, expansion of the lymphatic vessel was presented 2 weeks after the surgery to be continued to week. Neutrophil and lymphocyte were observed from 5 week (Fig. 5). Although overall edema was observed 2 weeks after the biopsy was performed from the tail base to the 50 mm distal part (C), the degree of edema began to be reduced from 3 week. Expansion of edema

and lymphatic vessel was presented until the end of the experiment, but the degree of expansion was insignificant when compared with the distal 18 mm region. Furthermore, although neutrophil and lymphocyte were discovered from 5 week of the experiment, the number was smaller when compared with the distal 18 mm region.

The cytoplasm of the endothelial cells of the blood vessels was observed to be brown according to the immunohistochemistry conducted on LYVE-1

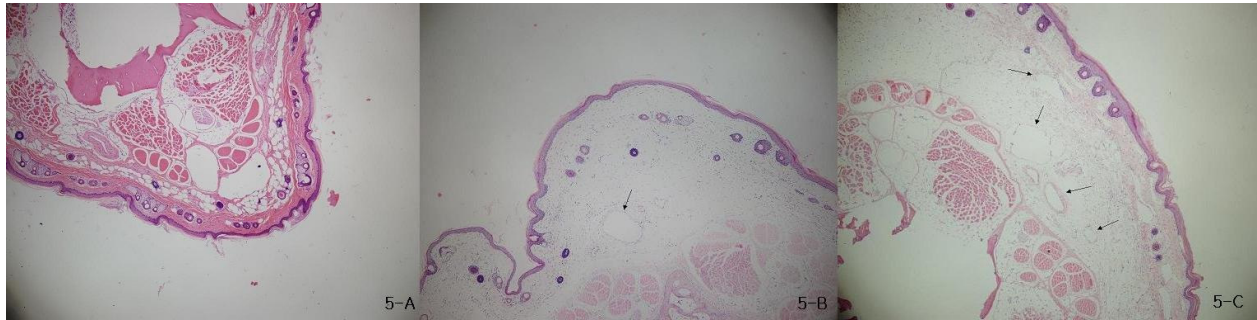


Fig. 5. Normal tail skin harvested 18 mm from the base of the tail shows thin epidermis and no dilated vessels in dermis (5-A, H&E stain x 40). Some dilated vessels in dermis (arrows) are seen at the groups of 2 and 4 weeks, respectively (5-B and 5-C, H & E stain x 40).

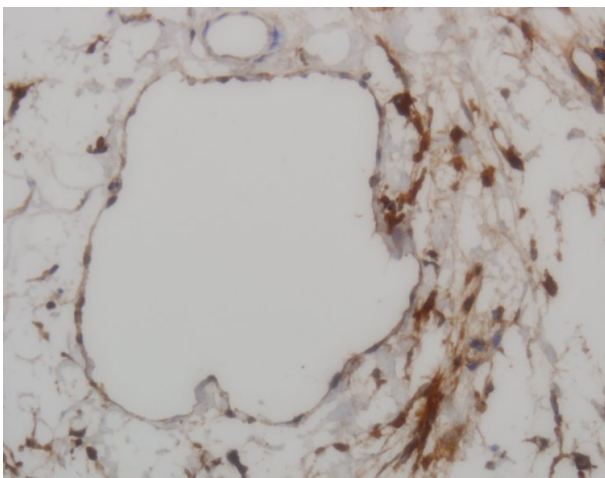


Fig. 6. Positive reaction of endothelial cell in dilated vessel is noted by lymphatic vessel hyaluronan receptor - 1 (LYVE - 1) stain. (x 400).

antibody to be verified as the lymphatic vessels (Fig. 6). Although positive reaction was presented in some cells around the lymphatic vessel, it was analyzed as a histiocyte or abnormal reaction as distinct lumen was not presented in such cells.

DISCUSSION

Although research on lymphedema requires understanding through pathophysiological analysis, experimental research remained inactive due to lack of experimental subjects. Hereupon, the treatment

method was also applied to patients based on theoretical possibility. Thus, although much time was passed since research was first conducted on lymphedema, the mechanism for histological changes, including the cause of lymphedema, remain unclear to perform treatment by using complex decongestive physiotherapy, a non - drug treatment. In this regard, the production of an appropriate experimental model will provide a new turning in the research and treatment of lymphedema.

Tabibiazar et al.⁷ and Swartz et al.^{8,9} used mouse tail to trigger lymphedema in animal models. Tabibiazar et al.⁷ made an incision in the skin 16 mm from the base part of the mouse tail and used the method of eliminating proximal tissue by using an instrument. On the other hand, Swartz et al.⁸ only performed surgical incision without removing the skin and tissue under skin in the surrounding area of the base part of the mouse tail. Also, Swartz et al.⁹ triggered lymphedema by using the method of transplanting abdominal muscle after binding the lymph vessel of the hips that begin from the tail of the mouse. As reproducibility of distinct lymphedema was insufficient in the application of methods used by Swartz et al.,⁸ this study introduced a new method of only removing the skin and tissue under skin in

the 5–10 mm area from the base part of the mouse tail without using instruments or complex surgical procedures. Thus, this study used a simple method to trigger lymphedema, verified the functional and histopathological changes within the animal body through volume change, histopathological change, and nuclear test. All test presented secondary lymphedema.

Advantages of this model are (1) easy to trigger lymphedema (It doesn't need instruments, complex surgical procedures or radiation), (2) have the advantages of cost-effectiveness, (3) and mice model have a great range of research tools available such as antibodies and various databases. Limitations of this model are (1) small-sized vessels compared with other animal model, (2) difficulty to monitor the transportation of substances, (3) and insufficient size for procedure as cannulation or complex surgery.

This study used LYVE – 1 for the immunological test of lymphedema. As the receptor existing within the lymphatic endothelial cell, it is used as the marker in lymphatic endothelial cells to be recently used in the immunohistological research of lymphedema and recognized as the most significant marker for lymphedema. Test was performed by using this immunological marker in the tail of the mouse that received surgery. In result, the stain around the lymphatic vessels was observed to verify lymphedema in the immunohistological aspect. However, stained LYVE – 1 was also presented in other surrounding tissue in addition to the lymphatic vessel. This is stipulated as a problem generated in the process of selecting an appropriate LYVE – 1 as there are slight difference in LYVE – 1 between humans and mice and various appropriate models exist for different types of experiments especially in case of mice.

The secondary lymphedema model of mouse verified in this study supports 3 important hypotheses of lymphedema. The first hypothesis is related with the relationship between lymphedema and infection. Similar to the study of Tabibiazar et al.,⁷ the research of authors presented a distinct volume increase after 1 week and displayed similar histopathological aspects. Although abnormal characteristics were not observed in the proximal region of surgery, partial edema was presented in the distal part of the mouse tail after 1 week and overall edema was presented after 2 week. Expansion of lymphatic vessels was observed with neutrophil and lymphocyte according to the passing of time. As inflammatory reaction has been reported in recent studies on lymphedema, it can be verified that the results of this study correspond to previously reported results.

Second, based on content related with the regional distribution, mouse tail edema must begin from the distal end part and must be most severe in this region when considering the Stopcock mechanism. However, according to the results presented in this study, lymphedema was not started from the distal part of the mouse tail. Also edema was presented in 2 week for generally distal parts that performed incision, with more distinct edema presented in the just distal part from performed incision. Thus, it is stipulated that these results support the regional filtration rate as the mechanism for secondary lymphedema than the Stopcock.

The last hypothesis is subfascial lymph drainage. Lymphedema triggered in mouse tail was alleviated without special treatment according to the passing of time. This is regarded to have been resulted from the changes in lymph discharge due to the removal of skin and tissue under skin during the surgical incision. In

other words, as the subfascial tissue does not receive any damage, it is stipulated that the decrease in lymph discharge of surface tissue damaged through surgical incision can be compensated according to the passing of time by the formation and anastomosis of new lymphatic vessels. Thus, it can be said that this study also supports the results of recent studies that have reported the greater role of subfascial lymph discharge in lymph discharge when compared with epifascial lymph drainage.

Along with recent research results, the most interesting part of this study was the fact that inflammatory reaction was presented even though lymphedema did not display any stimulations for triggering inflammatory reaction. In particular, the inflammatory reaction was transformed from an acute condition to a chronic condition according to the passing of time. Macrophage and increase in fibroblast, adipocyte, and keratinocyte are typically presented in acute lymphedema.¹⁰

Although the pathohistological results observed at the end of the experiment cannot accurately represent the aspects of chronic lymphedema presented in humans, increase in neutrophil and lymphocyte was presented to verify inflammatory reaction in lymphedema. Tabibiazar et al.,⁷ demonstrated with an experimental lymphedema model that dermis and subdermis exhibited intense inflammatory changes. The period that began to present inflammatory reaction with the passing of time is stipulated to hold greater significance than the period of the most distinct changes in volume. This does not signify that inflammatory reaction is simultaneously presented with volume changes according to lymphedema, but implies that inflammatory reaction is intensified according to the progress of lymphedema to present

the relationship of chronic lymphedema with inflammatory reaction. Although the acute and chronic periods cannot be clearly divided based on these results, it is stipulated that the 5th week after the surgical procedure can be judged as the period for dividing acute from chronic lymphedema when considering histological observations.

According to the increased awareness on lymphedema, the number of lymphedema patients desiring early treatment has increased recently. However, many patients still receive treatment at a later period as it is difficult to recognize lymphedema due to cancer surgery and various drugs used in the treatment process. Also, many patients pass the period of acute lymphedema as they delay their visit to the hospital due to various factors. As lymphedema in humans are differently classified as acute or chronic according to the time from occurrence to treatment, research must be conducted for both acute and chronic lymphedema.

This study classified the point of triggering lymphedema in mouse tail through surgical procedure to the next 4 weeks as the acute lymphedema period, and classified the period after the 5th week as the chronic lymphedema period. It is stipulated that this classification will be helpful in individual studies conducted on acute and chronic lymphedema.

The limitations of this study are as follows. First, as the experiment period was decided as 5 weeks, other pathohistological changes could not be verified afterward to present inadequate establishment of histological changes of chronic lymphedema. Second, although control group was formed to survive in the same environment, it is stipulated that more significant results can be produced by restricting the movement of the mouse tail. This is because there

is a possibility that the activities of the mouse tail affected the generation and improvement of lymphedema in the experiment group. Future studies on lymphedema according to quantity of motion must also consider movements of the tail. Third, although 5 – week – old female mice were used in the study, the mice presented differences in the length of the tail. Although the experiment group and control group both included the 70 mm area from the base to the distal part of the tail as the edema region, it is stipulated that minute differences could have originated from the changes in activities according to tail length.

Although this study holds significance in producing a lymphedema animal model based on a simple method than previous studies, different animal models must be produced in the future according to individual study on lymphedema.

Lymphedema can be generated by various causes, such as cancer therapy, injury, or congenital reasons. However, studies on the cause of lymphedema remain insignificant, and it is difficult to research lymphedema and apply new treatment methods as an appropriate research model has yet been developed. In this regard, the authors produced a lymphedema animal model that triggered acute and chronic lymphedema by using a new surgical method in the mouse tail, and verified its suitability as a research model through various tests. It is stipulated that easy and diverse approaches can be achieved as lymphedema was triggered through a simpler method than other studies by using an easily applicable animal. Further research

on the pathophysiological aspect of lymphedema can be expected with an improved approach for new therapeutic methods.

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