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# ALTERATIONS OF LYMPH FLOW AFTER LYMPHADENECTOMY IN RATS REVEALED BY REAL TIME FLUORESCENCE IMAGING SYSTEM

Y. Takeno, E. Fujimoto

Department of Nursing, Graduate School of Medicine, Nagoya University, Nagoya, Aichi Japan

*ABSTRACT*

*Secondary lymphedema is one of the sequella of cancer treatment that in inadequately understood. The purpose of the present study is to investigate lymphedema formation and to explore the escape routes for excess interstitial fluid using lymphadenectomy in a rat model. In twelve Wistar rats, lymph nodes in the right inguinal and popliteal fossas were completely removed and lymph vessels carefully ligated. After operation, treated hind limbs were evaluated by indocyanine green lymphography and circumferential measurement. Both evaluations were performed from day 3 to ten weeks. Approximately 2 to 3 weeks after operation, a network-like pattern of the fluorescent signal appeared around the surgical site which then transitioned into a linear pattern in the lower abdomen. Videorecordings identified fluorescent flow moving from the lower abdomen to the ipsilateral axillary lymph node and in some rats, the network-like pattern was also observed to pass transversely over the suprapubic region to the contralateral inguinal lymph nodes. The network-like pattern on the lower abdomen and the linear pattern to the axillary fossa were seen continuously to the end of observation. Circumferential measurements of the treated hind limbs increased initially and then declined over time. This imaging system may be useful to detect early changes in lymphatic flow before swelling occurs and further study is needed.*

**Keywords:** rats, ICG imaging, lymph flow, lymphadenectomy

Secondary lymphedema in non-tropical climes is most often caused by mechanical insufficiency of the lymphatic drainage system resulting from lymph node dissection performed to prevent tumor metastasis. These procedures lead to swelling of the extremity on the operated side. Treatments including surgical (e.g., lymph-venous anastomosis) and conservative (combined decongestive therapy, CDT) have been applied to mild, moderate, or intensely in severe cases (1). However, the detailed mechanism of lymphedema formation and the therapeutic action of CDT and its efficacy are still unclear (2). One of the reasons may relate to inaccurate measurement methods. Most previous studies have used limb circumferences and/or volume of an edematous limb for evaluation of improvement in lymphedema, since it has been difficult to visualize fluid accumulation (3) and escape route of excess interstitial fluid.

The fluorescence imaging method using indocyanine green (ICG), which is typically used for measuring liver function and blood vascular imaging, has recently been applied to the field of lymphography. ICG has the characteristic of exhibiting fluorescence after binding to albumin, both in blood plasma and the interstitial space. In the latter case, although ICG combined with albumin cannot be taken up by blood capillaries, it is absorbed into lymphatic capillaries, and thereby lymph vessels can be identified after subcutaneous ICG administration. Based on these properties of ICG, lymph flow can be observed transdermally in real-time by the ICG fluorescence imaging Photo Dynamic Eye (PDE) system (4). This system displays the superficial function of the lymphatic in an edematous region which cannot currently be detected by quantitative assessment methods (5). Using ICG lymphography, Yamamoto et al proposed a new severity staging system which consists of four patterns of lymph flow: linear, splash, stardust and diffuse (6). Linear is seen in normal and mild lymphedema cases, and the latter three are expressed as dermal backflow patterns based on the progression of lymphedema.

In order to understand and better improve therapy for lymphedema, it is important to understand lymphedema formation (7). Lymphedema studies using animals have an advantage because the models are easy to access, and also because it is possible to intervene during the study. In particular, one advantage is being able to administer chemical agents. We applied the PDE system after the subcutaneous administration of ICG to rats following lymphadetectomy with the purpose to investigate lymphedema formation and progression, and to search for an escape route for excess interstitial fluid as one aspect of fluid movement.

## *MATERIALS AND METHODS*

### *Materials*

Twelve male Wistar rats (aged 10-14 weeks) were used in the present study. All *in vivo* experiment protocols were reviewed and approved by the Animal Experiment Committee of Nagoya University.

### *Procedures*

Rats were induced with 4% isoflurane inhalation and maintained with approximately 1.6% isoflurane for operation. Bilaterally, the hind limbs were shaved in order to measure the circumferential measurement at both groins. Then, 0.2 ml of a 1.0 mg/ml solution of ICG was injected subcutaneously into the dorsum of the right paw and both the medial and lateral ankle to detect lymph nodes in the groin and lymph vessels in the femoral area. After normal lymph flow was confirmed by the photodynamic eye system (PDE: Hamamatsu Photonics K.K. Hamamatsu, Japan), which activates ICG by emitting light at the center wavelength of 760 nm, a circumferential incision from a surface of skin to subcutaneous tissue was made at the right groin. Lymph nodes and lymph vessels were then imaged by fluorescence and nodes with surrounding adipose tissue in the right groin and the ipsilateral popliteal fossa were completely excised. Fluorescent lymph vessels were carefully ligated under optical imaging using 10-0 monofilament non-absorbed suture. The skin edges were then sutured end to end by 4-0 nylon. No dressings or topical treatments were applied to any of the wounds.

### *Evaluation of Limb Edema*

Before operation, normal lymph flow was observed by ICG lymphography. After operation, the treated hind limb was evaluated by ICG lymphography and circumferential measurements taken. Both evaluations were performed at specific periods of time from day 3 post-operation to ten weeks.

For fluorescent lymphography, 0.2 ml of ICG was injected into the same sites as those used on the day of operation and observations were performed by PDE to examine dynamic lymph flow patterns. However, ICG was not injected if dermal back flow was already confirmed in the hind limb before the injec-tion. Fifteen minutes after the injection, fluorescent images visualized by PDE were video-recorded in digital format.

Limb circumferences of the operative

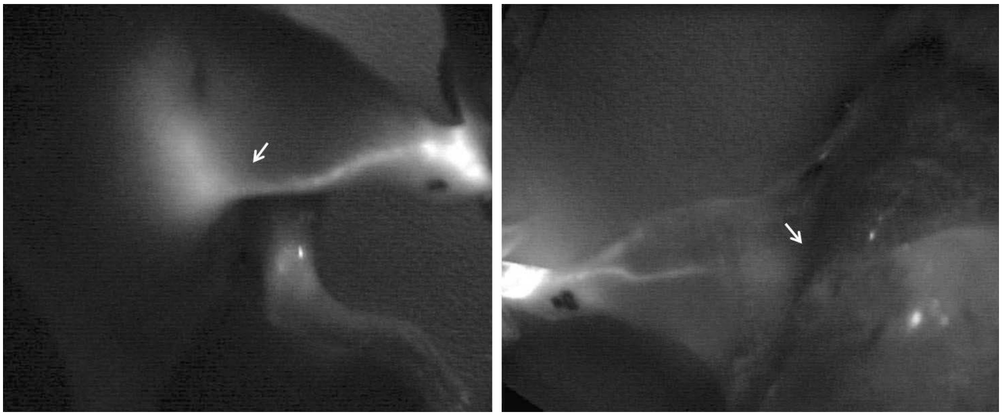

*Fig. 1. Preoperative images. Left: A linear fluorescent signal appears from the right ankle through the posterior crus to the popliteal fossa (arrow). Right: Another linear signal is found from the right ankle through the anterior thigh to the inguinal region (arrow).*

and contralateral sides were measured at the inguinal regions of both limbs with a soft plastic tape measure. Bilateral differences in the circumferences were represented as medians and ranges. The significance of differences was assessed with the non-parametric Wilcoxon single rank test with Bonferroni correction. Values of $p<0.05$ were considered significant.

## *RESULTS*

### *Preoperative Image*

All rats showed similar fluorescent images before operation (*Fig. 1*). The right foot pad including injection sites displayed intense fluorescence of ICG by PDE. From the right ankle, two linear fluorescent signals immediately appeared after the injection which and then reached the right popliteal fossa and the inguinal region, respectively. No fluorescent signal was observed on the proximal area of the inguinal region, nor on the other parts of the body except for the right hind limb.

### *Postoperative Images*

Lymph node excision and lymph vessel ligation at the inguinal region altered the fluorescent patterns observed before operation into different patterns. Results of the present study were categorized chronologically into three events: 1) abnormal images limited to the limb; 2) appearance of new flow patterns; and 3) stability of flow pattern.

### *Abnormal images limited to the limb*

The fluorescent signal did not show a linear pattern in any parts of the treated hind limb, but was distributed diffusely and/or made spotted fluorescent signals in all cases (*Fig. 2*). Such abnormal images were limited to the treated hind limb.

### *Appearance of new flow patterns*

New flow patterns of network-like and linear fluorescent signals appeared in all the rats. Such patterns appeared at week 1 in two of twelve rats, week 2 in five rats, and week 3 in five rats after operation, respectively.

A network-like pattern of the fluorescent signal was dense around the operative site from the anterior thigh to the lower abdomen. In addition, the fine and small network-like signal was depicted at one or several points in the lower abdomen and this became a linear pattern. All lines led to and reached the ipsilateral axillary fossa and accompanied

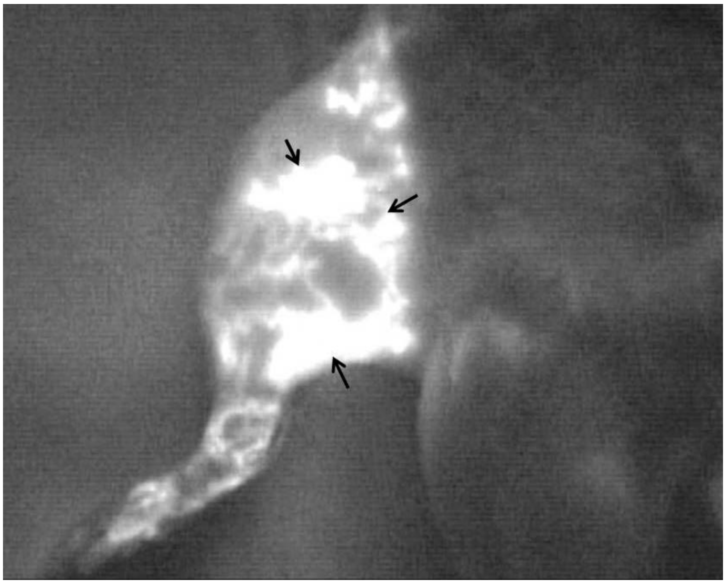

*Fig. 2. One week after operation. Dermal backflow is seen in the treated hind limb and the fluorescent signal displays as a stardust pattern (arrow).*

superficial epigastric and thoracoepigastric veins. With dynamic pictures by PDE, the fluorescent flow which moved from the lower abdomen toward the axillary fossa was identified. In nine of twelve rats, the network-like pattern of fluorescent signals was also observed to pass transversely over the suprapubic region to reach the contralateral inguinal fossa. A linear pattern was not seen in this area. The diffuse and spotted fluorescent signals remained in the anterior thigh even though the network-like and linear flow patterns appeared (*Fig. 3*). In the gluteal area and the dorsal region of the trunk, the network-like fluorescent signal was barely or never seen.

*Stability of flow pattern*

All rats demonstrated a new route to the ipsilateral axillary fossa beyond the incision line. When the new flow patterns began to appear, three rats had a single route to the ipsilateral axillary fossa while the remaining rats exhibited the additional route to the contralateral inguinal fossa. However, the route to the contralateral inguinal fossa disappeared in three rats during the observation period at week 2, week 5, and week 8 after operation, respectively. In addition to the three rats that demonstrated only a single route to the ipsilateral axillary fossa from the beginning, three more rats also displayed this single route to the axillary fossa by the end of the observation period (*Fig. 4*). Regarding the network-like pattern and the route to the ipsilateral fossa, there were a few variations among the rats, but each rat maintained one specific flow pattern at least until the end of the observation. Diffuse and spotted signals decreased with the passage of time, since the flow patterns were stabilized and completely disappeared around eight weeks after operation in all rats. The network-like pattern on the lower abdomen and the linear pattern to the axillary fossa was seen continuously to the end of observation. Lower fluorescent signals were seen in the gluteal and dorsal regions of the trunk, compared to the anterior thigh and the abdomen (*Fig. 5*).

*Circumferential measurements*

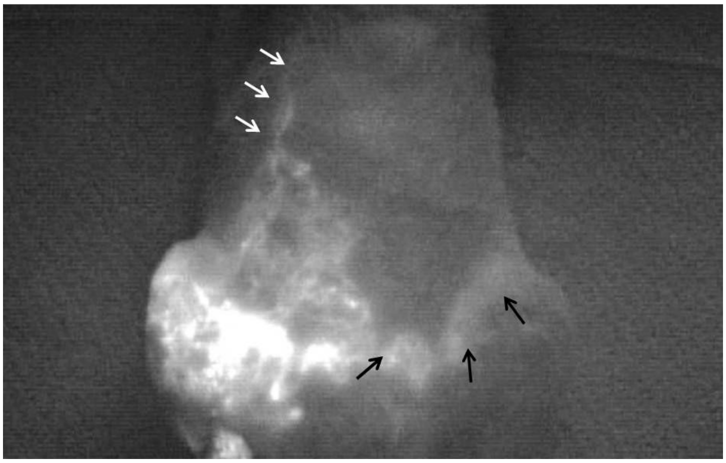

*Fig. 3. Three weeks after operation. New pathways are seen connecting to the ipsilateral axillary and the contralateral inguinal lymph nodes. A linear pattern is seen to the axillary lymph node (white arrows) and a network-like pattern from the right hind limb to the inguinal lymph node (black arrows).*

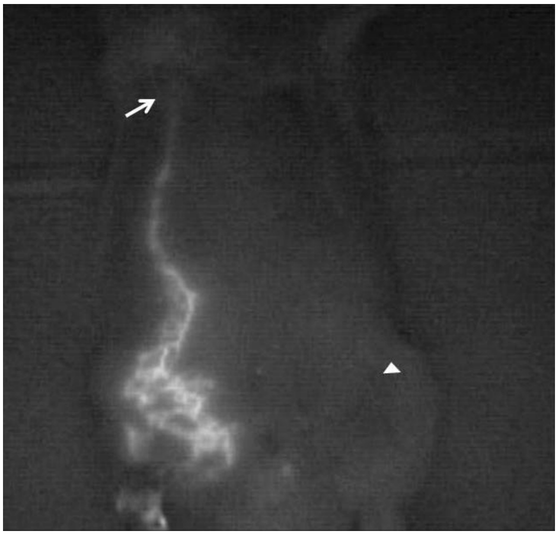

*Fig. 4. Eight week after operation. Typical pattern of single route to the ipsilateral axillary lymph node. (axillary lymph node- arrow; inguinal lymph node- arrowhead)*

Preoperative circumferential measurements of the right hind limbs were almost the same as in the left hind limbs in all rats. The median difference in circumference was 0 cm (from -0.1 to 0.2) in the preoperative period.

Circumference measurements of treated hind limbs increased soon after operation and achieved a peak on day 3 for three rats, week 1 for five rats, and week 2 for two rats. However, only two rats, whose maximum circumference measurement occurred in week 2, showed the same or smaller circumference in the right hind limb compared to the left, from day 3 for two weeks. The medians were 0.75 cm (from -0.2 to 1.9) on day 3 and 0.9 cm (from -0.2 to 2.6) on week 1, 0.55 cm (from -0.1 to 1.8) on week 2, respectively.

From week 3 to 10, there was a considerable variation in circumference changes among rats. In some rats, there was

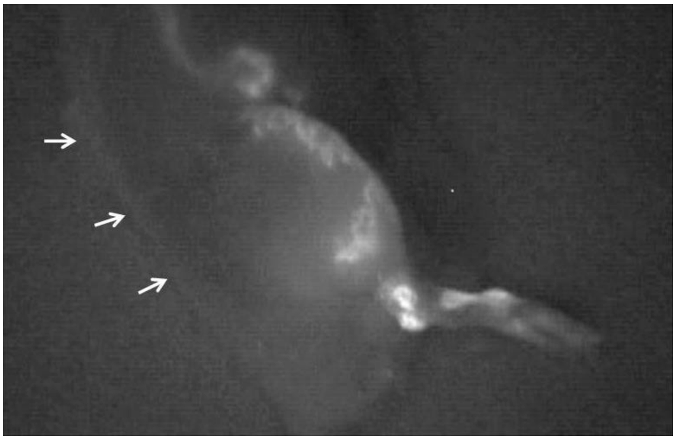

*Fig. 5. Less fluorescent signal is seen in the posterior thigh and the gluteal region with ICG imaging of the model. (vertebral column- arrows)*

a rapid reduction after the peak with a sustained plateau while others exhibited a continued mild reduction to close to the untreated hind limb. From weeks 3 to 10, the median difference decreased from 0.4 cm (from -0.4 to 1.2) to 0.1 cm (from -0.2 to 0.6).

*Statistical Analysis of Circumference Measurements*

Results revealed significant differences between the circumferences at pre-operative and day 3, and also between pre-operative and week 1 (both $p<0.05$). After week 3, there were no significant differences (*Fig. 6*).

*DISCUSSION*

In the present study using ICG and the PDE system, we have demonstrated that lymph node excision and vessel ligation at the inguinal site leads to development of collateral pathways to the ipsilateral axillary and contralateral inguinal lymph nodes. Dermal backflow images as a sign of lymphatic dysfunction and lymphedema appeared in treated hind limbs immediately post-operation and then gradually disappeared after collateral pathways were established. Therefore, collateral pathways are presumed to have a crucial role in improving lymphedema and for compensating for the lymphatic dysfunction from the operation. In particular, the pathway to the ipsilateral axillary lymph nodes may have a large potential for allowing lymph fluid to drain into the deep lymphatic system because lymph flow movement toward the ipsilateral axillary lymph nodes was continuously observed in the rats despite disappearance of the suprapubic pathway. This finding was first confirmed as real-time lymph movement using the PDE video-recording system. Lymph labeled by ICG was observed by film to vigorously move from the paw, beyond the incision, and to the ipsilateral axilla. Recently, dermal back flow was observed in lymphedema patients using ICG (8). Considering these results and our findings using PDP video, it is possible that the collateral pathway to the ipsilateral axillary lymph node may be activated in patients suffering from lower extremity lymphedema. This information would be beneficial to patients. However, other lymphatic imaging studies in humans with lymphedema have not shown this finding and perhaps this finding may be limited to the superficial imaging ability of our methods compared to the other methods which can image the deeper system. Superficial cutaneous lymph capillaries seen as network-like patterns were observed from

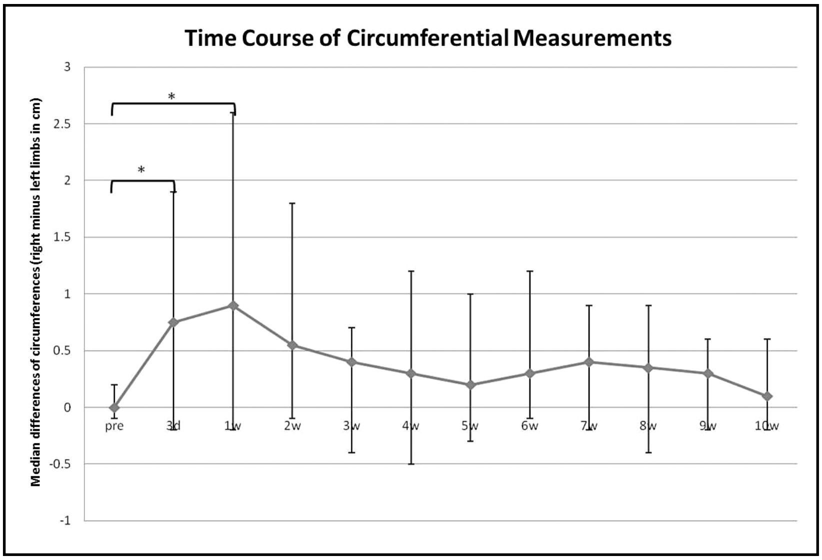


*Fig. 6. Mean difference in circumference increases to a peak at week 1 after operation (*p<0.05) and then gradually drops until week 10. (p>0.05).*

the thigh to the lower abdomen beyond the incision. Therefore, it is postulated that they also play a greater role in connecting the treated hind limb to the collateral pathway. Several studies found that the maximum spread of the visualized microlymphatic network was significantly larger in patients with lymphedema than in healthy controls (9,10) and this was thought to be a result of an impeded transport toward the deeper channels. However, these studies refer only to the capillary network from the injection site. In the present study, a spread of the superficial lymph network crossing the incision line was observed. We postulate that lymph capillaries and collectors regenerate and/or connect to each other and spread beyond the affected region where the lymph transport system is damaged. These capillaries might reach adjacent normal lymph collectors and then drain into normal lymph nodes.

The present study employed circumferential measurements in parallel with ICG lymphography. The statistical analysis shows that there are significant differences in the median difference of the circumferences between the preoperative period and day 3, and between the preoperative period and week 1. This coincides with the stage in which abnormal images are limited to the treated limb. The decline from week 2 overlaps the stage of the appearance of new flow patterns. Although severe dermal backflow patterns appeared in the treated right hind limbs of all rats, two rats showed smaller or almost the same circumferential measurements in the right hind limbs compared to the untreated left hind limbs until week 2. The treated right hind limbs gradually swelled, and then maintained the same circumferences as those in the left hind limbs at week 1 and 2 after operation. The circumferential measurements of the treated hind limbs, which were smaller than the untreated hind limbs until one week after operation, may have been influenced by the removal of adipose tissue at the time of operation, so the onset of swelling might be delayed in comparison with the emergence of dermal backflow. Although apparent swelling of the treated hind limbs was not identified until two weeks after operation, ICG lymphography revealed significant changes in lymph flow during this period. A similar finding has also been reported and discussed by Ogata et al (11). They found that swelling of the treated limb measured by circumference was not apparent until day 4 or 5 after lymph node resection, but that the ICG lymphography revealed significant changes in lymphatic vessels even at day 3. Therefore, for the early detection of lymphedema, it is important not to depend exclusively on visible swelling. ICG lymphography may thereby improve clinical practice substantially. In Ogata et al's study, only acute lymphatic damage from the preoperative period to day 7 post-operation was studied. We pursued lymphatic changes and swelling for 10 weeks. Although we chose to use just lymphadenectomy in rats to produce swelling for our 10 week time period to observe fluid and circumference changes, perhaps using the addition of radiation to the limb in the lymphedema model as first described by Kanter et al (12) would result in sustained lymphedema and this would be of interest to replicate our ICG imaging in that model to investigate the changes with sustained swelling and lymphatic dysfunction.

The results of the present study add valuable knowledge of lymphedema formation and progression and offer a promising way to understand an escape route for excess interstitial fluid from an edematous limb. Nonetheless, the applicability of our findings in this rat model to clinical lymphedema development after lymphadenectomy awaits further confirmation.

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**Yukari Takeno, MN**
**Department of Nursing**
**Graduate School of Medicine**
**Nagoya University**
**1-20, Daiko-Minami, Higashi-Ku**
**Nagoya, Aichi 461-8673, Japan**
**TEL: +81- 52-719-1504**
**FAX: +81-52-719-1568**
**E-mail: takeno.yukari@a.mbox.nagoya-u.ac.jp**