

PEDICLED VASCULARIZED LYMPH NODE TRANSFER TREATS LYMPHEDEMA IN RAT HIND LIMB: A SIMPLE EXPERIMENTAL STUDY DESIGN

I.O. Aydogdu, A. Demir, M.K. Keles, O. Yapici, L. Yildizy, Y. Demirtas

International Medicana Hospital, Department of Plastic, Reconstructive and Aesthetic Surgery (IOA), Samsun; Ondokuz Mayıs University, Faculty of Medicine, Department of Plastic, Reconstructive and Aesthetic Surgery (AD), Samsun; Diskapi Yildirim Beyazit Hospital, Department of Plastic, Reconstructive and Aesthetic Surgery (MKK), Ankara; Ondokuz Mayıs University, Faculty of Medicine, Departments of Nuclear Medicine (OY) and Pathology (LY), Samsun, and Lymphest Plastic Surgery Clinic (YD), Istanbul, Turkey

ABSTRACT

Vascularized lymph node transfer is a new and promising technique for the physiologic treatment of lymphedema and several clinical and experimental studies have been conducted in recent years. However, the exact mechanism of vascularized lymph node transfer is still unknown. In this study, we aimed to investigate treatment efficacy through the design of a simple and feasible experimental lymphedema model for testing a vascularized lymph node transfer technique. After a pilot study, 30 male Sprague-Dawley rats were divided into two groups and lymphedema was induced in the hindlimb of both groups. In Group 1 (control) (n=15), no treatment was applied while pedicled lymph node transfer was applied in Group 2 (experiment) (n=15). Model dynamics were assessed with lymphoscintigraphy, limb measurement, and histological analysis. A statistically significant limb circumference reduction at the ankle was seen on days 30 and 90 in Group 2 (24.4 ± 1.05 , 23.86 ± 0.83 mm) as compared to Group 1 (25.93 ± 1.38 and 25.4 ± 1.12 mm) ($p < 0.05$), and lymphatic tracer transport improved in 13 out of the 15 animals in

Group 2. A statistically significant reduction in histological scores was achieved in Group 2 ($p < 0.05$). In this rat hindlimb lymphedema model, our vascularized lymph node transfer technique is an effective physiologic surgical treatment and represents a feasible experimental model for future studies.

Keywords: lymphedema, rat model, treatment, lymph node transfer, lymphoscintigraphy

The lymphatic system, one of the least understood structures of the human anatomy, is a network formed of thin vessels working like a vacuum with low hydrostatic pressure. The function of this system is to return fluid and plasma which has leaked into the interstitial space from blood capillaries back into the blood circulatory system (1). The system also performs a protective filter function against foreign body invasion as part of the immune system (2).

Lymphedema is the collection of lymphatic fluid that is rich in protein in the intercellular space, which presents as swelling and results from an imbalance between net blood capillary filtration and lymphatic return (1).

While physiologic operations in lymphedema therapy aim to reconstruct the lymphatic drainage system, excisional interventions aim to shrink the extremity by removing cutaneous and subcutaneous tissue. Free lymph node transfer is one of the physiologic operations aiming to reconstruct the lymphatic system.

In this study, we pursued a simple lymphedema model and evaluated the results of a lymph node transfer technique.

MATERIALS AND METHODS

Pilot Study

Three male Sprague-Dawley rats weighing 245-320 g were used. All of the procedures were performed in accordance with the guidelines of the Animal Research Committee of Ondokuz Mayıs University in Samsun, Turkey. Intraperitoneal (i.p.) ketamine at 100 mg/kg and intramuscular xylazine at 35 mg/kg were used for anesthesia in all procedures. A surgical microscope (316, OPMI-99, Zeiss, Germany) and standard microvascular instruments were used. The inguinal region was shaved, prepared, and draped while observing the principles of sterility. Each rat was fixed on the operating tray in the supine position. A superficial epigastric artery-based rectangular inguinal fat flap was dissected (*Fig. 1A*) and excised, and it was then sent for a pathological examination for lymph nodes. The specimens were separately fixed in a neutral formalin solution with 10% buffer for 48 hours, dehydrated in alcohol, and embedded in paraffin blocks. Sections of 5 micrometers were cut, deparaffinized, and stained with hematoxylin and eosin (H&E). The tissue samples were examined with a standard light microscope to confirm the presence of lymph nodal tissue in the inguinal dissection material (*Fig. 1B*).

Lymphedema Model

As described in the pilot study section, the pedicled inguinal lymph node flap was harvested in all animal groups. After elevation, the flap was buried under the undermined abdominal skin to maintain the tissue (*Fig. 1C*). At the same time, a 1-cm wide skin strip was excised from the inguinal region, and the skin edges were sutured to the muscles to protect the skin gap (*Fig. 1D*). An additional lymph node excision was made in the popliteal region. At the end of day two, circumferential edema occurred in all animals. To prevent epithelialization, we scratched the healing tissue once every three days to achieve a stable lymphedema in this model.

Study Design

In this study, 30 male Sprague-Dawley rats, weighing 245-320 g, were divided into two groups. In Group 1, only the lymphedema-provoking surgery was performed. In Group 2 (treatment group), after the lymphedema-provoking surgery, the lymph node was buried under the abdominal skin. After completion of the procedure, each rat was returned to its individual cage with free access to food and water. Acetaminophen (50 mg/kg i.p.) and cefazoline (10 mg/kg i.p.) were administered for postoperative analgesia and infection prevention. At the end of the first week, the buried lymph node flap was dissected and transferred distal to the de-epithelialized area on the leg without allowing for epithelialization. As in the original model, we hindered the epithelialization process every three days to maintain lymphedema in the leg.

Limb Diameter Measurements and Lymphoscintigraphy

Each extremity of the animals was quantitatively measured using a measuring tape on days 0, 2, 10, 15, 30, and 90. The measurement was made at a single point at the level of the ankle region.

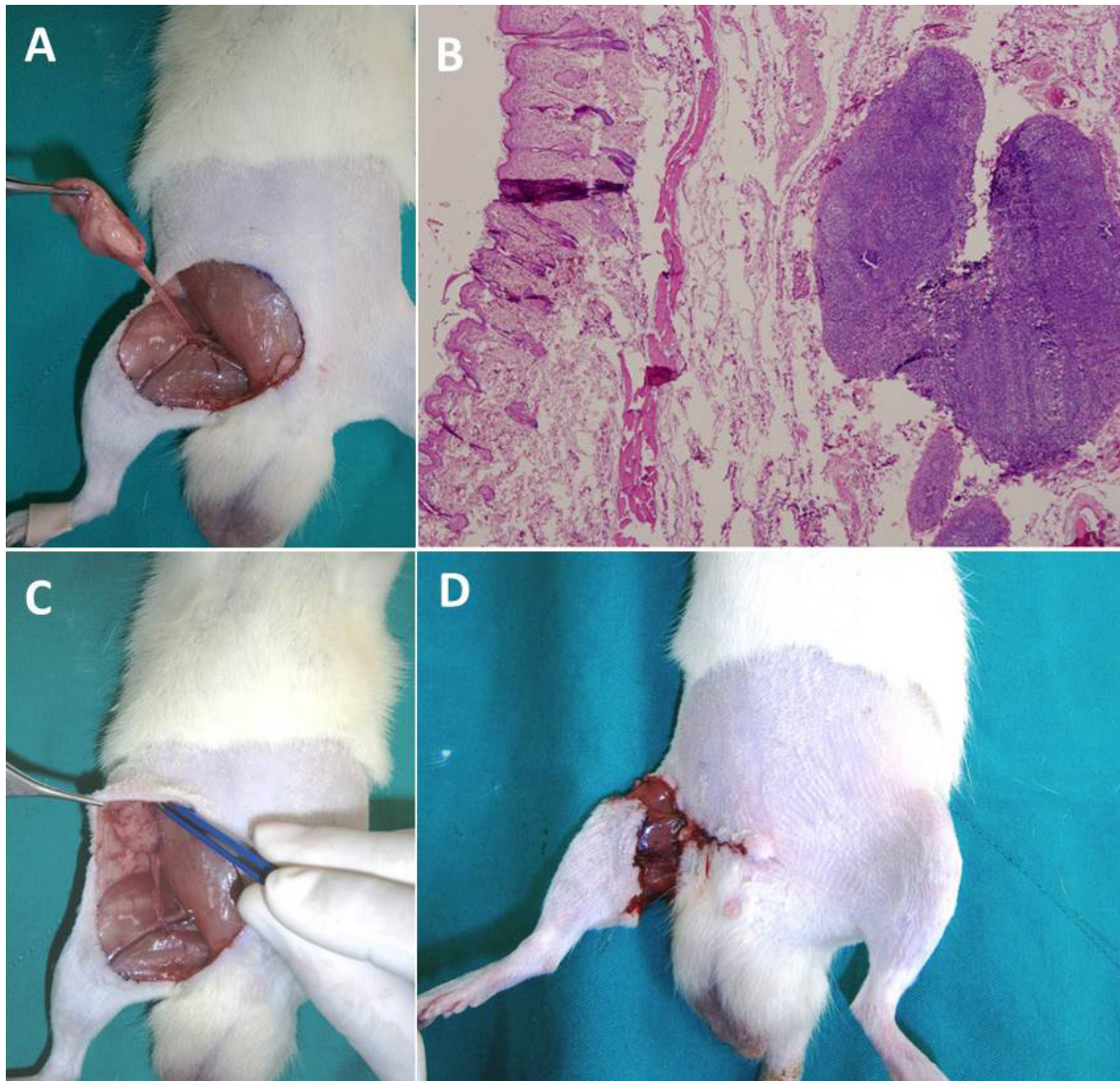


Fig. 1. Lymphedema model development and initiation. The model utilizes a superficial epigastric artery-based rectangular flap which is elevated from the inguinal region (A) and histologically demonstrates the presence of nodal tissue using H&E staining (B). This flap was temporarily buried under abdominal skin while the lymphedema developed (C) from popliteal node removal and a 1-cm wide skin strip circumferentially excised with skin edges sutured to the muscles to protect the skin gap (D).

Lymphoscintigraphy, an imaging technique to identify the lymph drainage, was performed on days 5 and 20 after surgery by using an ADAC Medical Systems Vertexplus EPIC dual head Gamma Camera and Pegasys Computer (Philips® Medical Systems, Netherlands). Before lymphoscintigraphy,

all animals were anesthetized as described previously and 0.02 mCi ^{99m}Tc (technetium) nanocolloid was injected intradermally dermis between the first and second toe. All procedures were performed in the supine position, and a whole body scan was taken at 0, 3, and 30 minutes.

TABLE 1
Ankle Measurements of Legs in Control Group (Group 1)
and Lymph Node Transfer Group (Group 2)

Day	Group 1		Group 2	
	operated leg	non-operated leg	operated leg	non-operated leg
0	22.66 ± 1.11	22.69 ± 0.22	22.93 ± 0.79	22.9 ± 0.12
2	27.73 ± 1.62	22.19 ± 0.1	27.88 ± 1.14	22.91 ± 0.1
10	27.13 ± 1.40	22.28 ± 0.16	27.26 ± 11.16	22.88 ± 0.21
15	26.4 ± 1.35	23.01 ± 0.99	26.23 ± 1.18	22.60 ± 0.74
30	25.93 ± 1.38	22.98 ± 0.49	24.4 ± 1.05	22.9 ± 0.55
90	25.4 ± 1.12	22.88 ± 0.51	23.86 ± 0.83	23.06 ± 0.15

Histological Analysis

From the inguinal region starting from proximal border of the de-epithelialized area and extending to the distal end of the transferred lymph node flap, 3×3-cm specimens, including the skin, fat tissue, lymph nodes, and the muscle, were obtained for histological studies at the 90 day time point before lymphedema started to subside. Specimens were separately fixed in a neutral formalin solution with 10% buffer for 48 hours, dehydrated in alcohol, and embedded in paraffin blocks. Sections of 5 micrometers were cut, deparaffinized, and stained with H&E. Tissue samples were examined with a standard light microscope.

Three different parameters were examined using H&E staining of the tissue samples: lymphatic ectasia, stasis, and intracellular edema. One experienced pathologist examined these findings to define the histological scores. All specimens from each subject were examined and scored semi-quantitatively as 0, 1, 2, or 3. The maximum score was 3, indicating maximum lymphatic ectasia, stasis, and intracellular edema of the tissue.

Statistical Analysis

Comparisons of data among the control

and experimental groups were performed using the Student's t-test and chi-square test. A $p < 0.05$ was used to determine a significant differences.

RESULTS

Two rats died due to infection during the study. These were replaced with new subjects and the same surgical protocol and timing was applied to new animals. All animals had a stable weight during the study.

Limb Diameter Measurements

The limb diameter measurements of the groups at the time points are shown in *Table 1*. At the ankle level, the difference between the lymph node transfer and control groups was statistically significant on days 30 and 90, respectively ($p < 0.05$) (*Figs. 2, 4A*). The non-operated limb diameter measurements in both groups showed no statistically significant changes during the 90 day follow-up.

Lymphoscintigraphy

On the 20th day, lymphatic drainage occurred in only 2 of 15 animals in the control group at the 30 minute time point. In contrast, lymphatic drainage occurred in

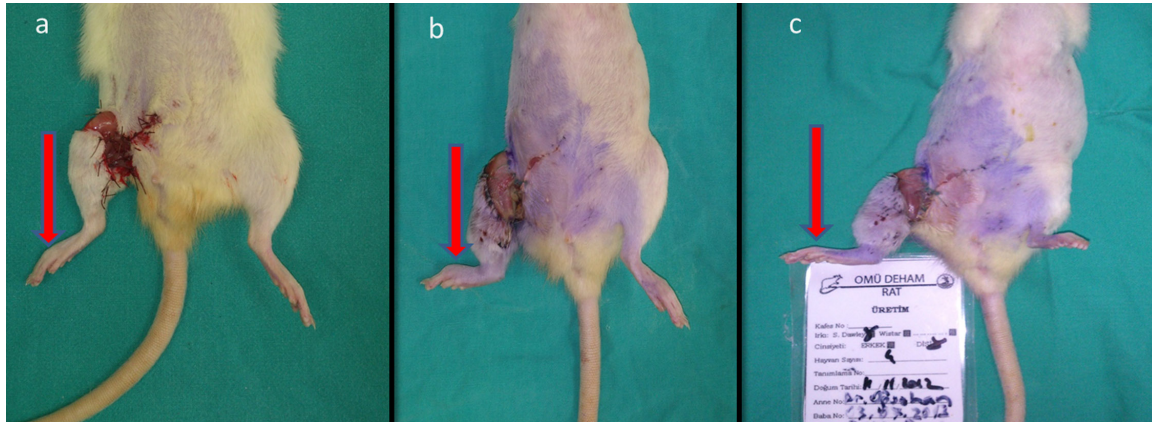


Fig. 2. Serial pictures of a rat during the cycle of edema formation and resolution. Immediately after operation (a) and day 7 after excision of skin and lymph node dissection (b). Note that the swelling on the foot dorsum decreases after lymph node transfer (c).

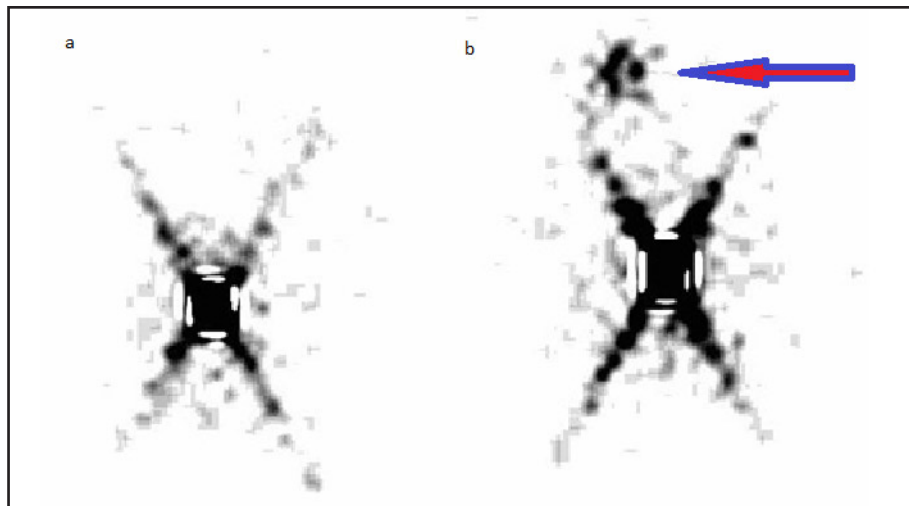


Fig. 3. Lymphoscintigraphic images of typical rat on day 20. In the control group (a), there is no radionuclide drainage 30 minutes after injection. In the experimental group (b), the arrow shows radionuclide uptake in the liver, which indicates effective tracer transport from the foot injection site to the central blood circulation.

13 of 15 animals in the lymph node transfer group (Figs. 3 and 4B). The difference between the control and lymph node transfer groups was statistically significant ($p < 0.05$).

Histological Analysis

At the 90 day time point, the lymph node transfer group demonstrated mean lymphatic

ectasia, stasis, and intracellular edema scores of 1 ± 0.53 , 0.86 ± 0.63 , and 1.4 ± 0.73 , respectively (Fig. 4C). The control group demonstrated mean lymphatic ectasia, stasis, and intracellular edema scores of 2.2 ± 0.41 , 2.4 ± 0.73 , and 2.6 ± 0.73 , respectively (Fig. 4C). All three histologic scores were statically lower in the lymph node transfer group than in the control group ($p < 0.05$).

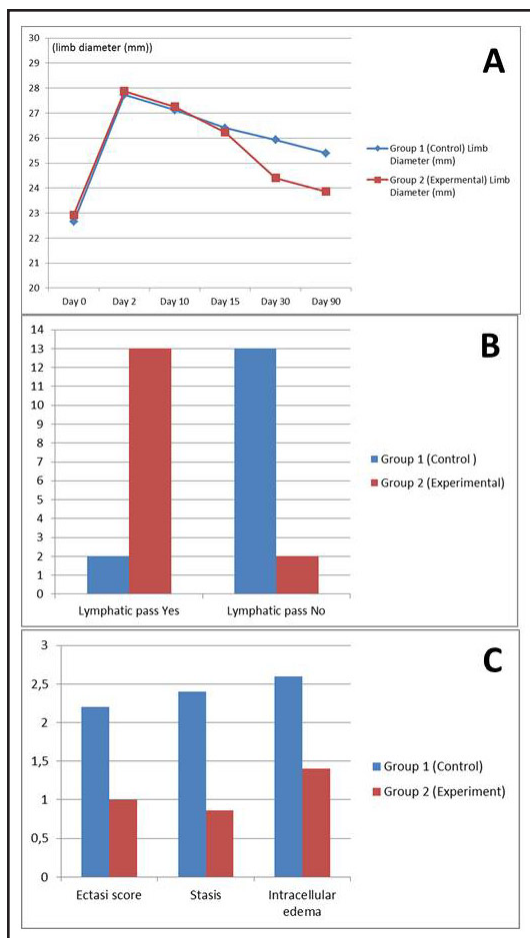


Fig. 4. Model measurement parameters demonstrating (A) decreased mean ankle circumference on days 0 to 90, which were statistically significant at days 30 and 90 between the groups; (B) an increased tracer transport to the central blood circulation in Group 2; and (C) decreased mean lymphatic ectasia, stasis, and intracellular edema scores in Group 2.

DISCUSSION

Lymphedema is generally regarded as incurable (3), and treatment modalities primarily consist of physiotherapy and surgical procedures. Surgical procedures have some advantages although there are a limited number of surgical techniques (4). These procedures can be handled in two ways: excisional and physiologic. Physiologic

operations are further divided into lympho-lymphatic anastomosis, lympho-venous anastomosis, and vascularized or non-vascularized lymph node transfer.

In recent years, vascularized lymph node transfer has gained popularity in clinical practice (5-9). It is a relatively new and devolving technique (10). However, the exact mechanism of this procedure has not yet been completely understood. Possible mechanisms are the reconnection of afferent and efferent lymphatics, lymphangiogenesis, and/or shunting (3,6). In a recent study, Cheng et al reported that lymph node transfer can construct channels connecting lymphatic vessels to the venous system (10). This mechanism is also supported by a recent clinical study (11). Other clinical studies have reported success with vascular lymph node transfer (5,7,8). We propose from our current study that the statistically significant difference may be related to the transferred lymph node in Group 2, which could act as a drainage channel for the lymphatic fluid to reach the venous system. Another recent study revealed that higher numbers of lymph nodes can function even more efficiently. In our study, we elevated the entire inguinal subcutaneous tissue as a flap with no regard for the number of lymph nodes and the size of the subcutaneous tissue. Although precise localization is controversial, we transferred the lymph node flap to the proximal area of the lymphedematous leg. Due to the short length of the pedicle, we could not transfer the flap to distant parts of the leg. A major limitation of our model is that it does not simulate the clinical situation where a lymph node is taken from one area of the body with its vascular supply and transplanted to another area. In our model, the pedicle flap blood supply and return was also not disrupted when it was translocated to the new area.

The physiological surgical techniques used in lymphedema treatment are microsurgical and supermicrosurgical procedures. In lympho-lymphatic and lympho-venous

shunt operations, the lymphatic vessels are less than 1 mm in diameter, which makes this technique difficult to perform (12). Vascularized lymph node transfer also requires advanced microsurgical equipment and skills. In animal studies, the technique becomes harder due to the small anatomical structures, and it requires a consistent, simple, and feasible lymphedema and vascularized lymph node transfer model. Lymphedema represents increased fluid and protein in the intercellular space, and this lymph imbalance can cause the number of fibroblasts, adipocytes, and keratinocytes to increase in the skin and subcutaneous tissue (13). It is impossible to say that the animal models where lymphatic drainage is blocked surgically can properly mimic lymphedema in patients. Nevertheless, there are some lymphedema and vascular lymph node transfer models described in the literature that more closely mimic clinical lymphedema (3,13-22). In our study, we aimed to create a simplified model for lymph node transfer.

From an historic point of view, Drinker et al managed to induce sustained experimental lymphedema in a dog for the first time in 1934 (13). In 1968, Olszewski et al induced lymphedema in a dog's thigh by performing a circumferential skin excision, subcutaneous tissue excision, and lymph node and lymphatic duct excisions (19). Thus, they described a consistent model that could be applied to other animals to induce lymphedema. The first lymphedema model in a rat was achieved by Wang et al in 1985 (22). Similar to the lymphedema model introduced by Olszewski et al in a dog, Wang et al managed to successfully induce lymphedema in the hind leg of a rat through a lymphatic tissue excision, popliteal lymph node excision, and circumferential skin tissue cut. Therefore, they defined the first lymphedema model on the hindlimb of a rat (19,22).

The clinical application of vascular lymph node transfer was first reported by Clodius et al (23), and the first vascularized lymph node transfer model was introduced

by Shesol et al in 1979 (20). Today, there is a limited number of vascular lymph node transfer models described in the literature (3,14-18,20,21).

In our study, we induced lymphedema using a method similar to that defined by Wang et al except that we did not excise the inguinal lymph node in the same way (22). We removed the skin comprising the inguinal lymph nodes over the superficial epigastric artery as a flap, just as Shesol et al did in their study (20). Following the aforementioned method and by performing a circumferential skin incision, we obstructed the lymphatic flow of the skin, and we contributed to this by leaving a gap of 1 cm between the distal and proximal skin ends. We also dissected the popliteal lymph nodes. We preserved the 1-cm gap between the skin ends to prevent epithelialization and worked actively to keep it denuded, which differed from Wang et al's technique. Wang et al generated lymphedema in a rat model spanning 270 days without irradiating the tissues. Unlike in our study, lymphedema decreases between the 30th and 90th days in Wang's model and then increases in 180th and 270th days. In our model we established a stable lymphedema between 30th and 90th days (22). We, therefore, prefer to sacrifice the animals on the 90th day before lymphedema subsides. This timing may be a limitation of the current model, and pre-existing lymphatics and lymphatic regeneration in deep tissue lymphedema may likely be important factors which prevent longer duration of the lymphedema in our model (22). In previous studies, Kanter et al and Lee-Donaldson et al demonstrated that irradiated models are more consistent when compared to non-irradiated models (24,25). In our model we did not irradiate the leg to minimize the tissue damage which might affect the flap viability both in pre-transfer and post-transfer period via pedicle damage. This choice is a model limitation for sustained lymphedema but it allows for pedicle protection.

Lymph node transfer with the superficial epigastric artery's vascular pedicle was

performed in the popliteal area following a lymphadenectomy by Shesol et al but it was not done in the inguinal area (20). In our study, the inguinal lymph nodes were removed based on the superficial epigastric artery and placed under the abdominal skin to create lymphedema. After lymphedema appeared, the lymph node flap was removed from this area and transferred to the inguinal area proximal to the swelling using a technique that did not require microsurgery. Therefore, although we did not face the difficulties of microsurgery, this model is not reflective of a vascularized lymph node transfer in the clinic. In addition to the sustainability of lymphedema in Group 1, it was shown that the lymphedema model was successful. Furthermore, Shesol et al did not wait to achieve lymphedema in their study. In our study, we transferred the vascular lymph node flap after lymphedema formation, which mimics the clinical condition more accurately.

Most vascular lymph node transfer models require a free tissue transfer (3,14-18,21). When it is combined with the hind-limb lymphedema model, the pedicled lymph node transfer is an easy alternative technique to the procedures that require microsurgical techniques. This flap model can also be used as a free tissue transfer. The femoral artery can be added to the pedicle so that a wider vessel diameter can be achieved. The pedicle length of the flap can be elongated up to 3 cm with the dissection of the femoral artery.

This experimental study may also be utilized in the future to determine whether the vascular lymph node transfer procedure may provide a shunt between the lymphatic vessels and the venous system using different imaging techniques.

In Group 1, which did not receive the vascular lymph node transfer, the nanocolloid appeared in the liver as an indication of transfer to the systemic circulation in 2 of the 15 rats at the 30 minute time point. Despite our best efforts to preserve the circumferential skin excision site, a superficial lymphatic flow

might have developed in the remaining granulation tissue. In Group 2, we showed that the Tc-99-labeled nanocolloid appeared in the liver on the 20th day following the vascular lymph node transfer in 13 of the 15 rats at 30 minutes, which was statistically significant. In this group, the reason why lymphatic drainage to systemic circulation did not occur in 2 of the 15 rats might be due to the infection and fibrosis of the surgical area. Although all rats received antibiotic treatment and although daily dressings were replied during the study, these two rats had an infection in their open wounds. This complication be due to an inadequate dosage or some factor outside the action spectrum of the drug. We did not image at later time points and perhaps these two subjects would have shown eventual transport to the central circulation (as possibly with more in the control group) if we continued to later time points.

CONCLUSION

We conclude that when combined with this hindlimb lymphedema model created by a skin excision and lymph node dissection, the superficial epigastric artery-based groin fat flap is a consistent and easy surgical procedure to simulate lymph node transfer. This combined model can provide an opportunity for researchers to investigate mechanistic aspects of vascularized lymph node transfer physiology in future studies.

REFERENCES

1. Ramos, SM, LS O'Donnell, G Knight: Edema volume, not timing, is the key to success in lymphedema treatment. *Am. J. Surg.* 178 (1999), 311-315.
2. Pan, WR, H Suami, GI Taylor: Lymphatic drainage of the superficial tissues of the head and neck: Anatomical study and clinical implications. *Plast. Reconstr. Surg.* 121 (2008), 1614-1624; Discussion 25-26.
3. Kwiecien, GJ, S Uygur, J Korn, et al: Vascularized axillary lymph node transfer: A novel model in the rat. *Microsurgery* 35 (2015), 662-667.

4. Tervala, TV, P Hartiala, T Tammela, et al: Growth factor therapy and lymph node graft for lymphedema. *J. Surg. Res.* 196 (2015), 200–207.
5. Becker, C, J Assouad , M Riquet, et al: Postmastectomy lymphedema: Long-term results following microsurgical lymph node transplantation. *Ann. Surgery.* 243 (2006), 313-315.
6. Cheng, MH, SC Chen, SL Henry, et al: Vascularized groin lymph node flap transfer for postmastectomy upper limb lymphedema: Flap anatomy, recipient sites, and outcomes. *Plast. Reconstr. Surg.* 131 (2013), 1286-1298.
7. Cheng, MH, JJ Huang, DH Nguyen, et al: A novel approach to the treatment of lower extremity lymphedema by transferring a vascularized submental lymph node flap to the ankle. *Gyn. Oncol.* 126 (2012), 93-98.
8. Lin, CH, R Ali, SC Chen, et al: Vascularized groin lymph node transfer using the wrist as a recipient site for management of postmastectomy upper extremity lymphedema. *Plast. Reconstr. Surg.* 123 (2009), 1265-1275.
9. Saaristo, AM, TS Niemi, TP Viitanen, et al: Microvascular breast reconstruction and lymph node transfer for postmastectomy lymphedema patients. *Ann. Surg.* 255 (2012), 468-473.
10. Cheng, MH, JJ Huang, CW Wu, et al: The mechanism of vascularized lymph node transfer for lymphedema: Natural lymphaticovenous drainage. *Plast. Reconstr. Surg.* 133 (2014), 192e-198e.
11. Patel, KM, CY Lin, MH Cheng: From theory to evidence: Long-term evaluation of the mechanism of action and flap integration of distal vascularized lymph node transfers. *J. Reconstr. Microsurg.* 31 (2015), 26-30.
12. Demirtas, Y, N Ozturk, O Yapici, et al: Comparison of primary and secondary lower-extremity lymphedema treated with supermicrosurgical lymphaticovenous anastomosis and lymphaticovenous implantation. *J. Reconstr. Microsurg.* 26 (2010), 137-143.
13. Shin, WS, A Szuba, SG Rockson: Animal models for the study of lymphatic insufficiency. *Lymph. Res. Biol.* 1 (2003), 159-169.
14. Andrzejewski, W, KW Zielinski: Method for vascularized lymph nodes transplantation in rats. *Zeitschrift fur experimentelle Chirurgie, Transplantation, und kunstliche Organe : Organ der Sektion Experimentelle Chirurgie der Gesellschaft fur Chirurgie der DDR.* 20 (1987), 192-196.
15. Can, J, R Cai, S Li, et al: Experimental study of lymph node auto-transplantation in rats. *Chin. Med. J.* 111 (1998), 239-241.
16. Chen, HC, BM O'Brien, IW Rogers, et al: Lymph node transfer for the treatment of obstructive lymphoedema in the canine model. *Br. J. Plast. Surg.* 43 (1990), 578-586.
17. Francois, CG, PC Brouha, LA Laurentin-Perez, et al: Vascularized lymph node transplantation induces graft-versus-host disease in chimeric hosts. *Transplantation* 81 (2006), 1435-1441.
18. Nguyen, DH, PY Chou, YH Hsieh, et al: Quantity of lymph nodes correlates with improvement in lymphatic drainage in treatment of hind limb lymphedema with lymph node flap transfer in rats. *Microsurgery* 6 (2016), 239-245.
19. Olszewski, W, Z Machowski, J Sokolowski, et al: Experimental lymphedema in dogs. *J. Cardiovasc. Surg.* 9 (1968), 178-183.
20. Shesol, BF, R Nakashima, A Alavi, et al: Successful lymph node transplantation in rats, with restoration of lymphatic function. *Plast. Reconstr. Surg.* 63 (1979), 817-823.
21. Uygur, S, C Ozturk, M Bozkurt, et al: A new vascularized cervical lymph node transplantation model: An anatomic study in rats. *Ann. Plast. Surg.* 71 (2013), 671-674.
22. Wang, GY, SZ Zhong: A model of experimental lymphedema in rats' limbs. *Microsurgery* 6 (1985), 204-210.
23. Clodius, L, PJ Smith, J Bruna, et al: The lymphatics of the groin flap. *Ann. Plast. Surg.* 9 (1982), 447-458.
24. Kanter, MA, SA Slavin, W Kaplan: An experimental model for chronic lymphedema. *Plast. Reconstr. Surg.* 85 (1990), 573-580.
25. Lee-Donaldson, L, MH Witte, M Bernas, et al: Refinement of a rodent model of peripheral lymphedema. *Lymphology* 32 (1999), 111-117.

Musa Kemal Keles, MD
Diskapi Hastanesi Plastik Cerrahi AD
Ankara, Turkey
Fax: 90 (332) 263 10 50
Phone: 90 506 735 3553
E-mail: mukeke@gmail.com.tr