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Lymphaticovenous anastomosis in rabbits: A novel live experimental animal model for supermicrosurgical training

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Abstract *Background:* Lymphaticovenous anastomosis is widely used in lymphedema management. Although its effectiveness in reducing edema in patients can be clinically observed, evaluating the long-term outcomes of this technique can be complex. This study established an animal model to assess the outcomes of lymphaticovenous anastomosis technique at 15 and 30-days post-surgery using indocyanine green lymphography, Patent Blue V dye injection, and histopathological examination.
Methods: An experimental model was established in the hindlimbs of 10 rabbits using the popliteal vein and afferent lymphatic vessels in the popliteal area. The subjects were divided into two groups: the first group (n = 5) underwent patency assessment at 0 and 15 days, and the second group (n = 5) at 0 and 30-days, resulting in 20 anastomoses. Patency was verified at 0, 15, and 30-days using indocyanine green lymphography and Patent Blue V injection. Histopathological examinations were performed on the collected anastomosis samples.
Results: The patency rate was 90% (19/20) initially, 60% (6/10) at 15 days post-surgery, and 80% (8/10) at 30-days. The average diameter of lymphatic vessels and veins was 1.0 mm and 0.8 mm, respectively. The median number of collateral veins was 3; the median surgical time was 65.8 min. Histopathology revealed minimal endothelial damage and inflammatory responses due to the surgical sutures, with vascular inflammation and thrombosis in a single case. Local vascular neoformations were observed.

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Conclusion: This study highlights the reliability and reproducibility of using rabbits as experimental models for training in lymphaticovenous anastomosis technique owing to the accessibility of the surgical site and dimensions of their popliteal vasculature.

Lymphedema is a chronic pathological condition characterized by the extensive stagnation of lymphatic fluid, inflammatory responses, and deposition of fibroadipose tissue.[1] Progressive accumulation of adipose tissue and fibrotic changes in the affected area lead to noticeable increase in limb volume, sensation of heaviness, impaired functionality, heightened vulnerability to infections, and secondary tumor development.[2] These factors are detrimental to the patients' overall quality of life and negatively affects their functional capabilities, social interactions, and psychological well-being.[3,4]

Conventional management of lymphedema relies on conservative and nonsurgical interventions.[5,6] Nonetheless, the implementation of microsurgical techniques such as lymphaticovenous anastomosis (LVA) and vascularized lymph node transfer has demonstrated notable efficacy in reducing limb volume, mitigating cellulitis risk, reducing reliance on compression garments, and minimizing the need for cellulitis treatment.[6,7]

Several studies have attempted replicating LVA in live experimental animal models to facilitate surgeon training. However, to our knowledge, only a few training models have been reported for LVA. Most procedures conducted in live experimental animal models involved the hindlimbs of dogs,[8,9] connection between saphenous lymphatic vessels and saphenous veins in pigs,[10] and linking of peritoneal lymph ducts with iliolumbar veins,[11-13] and saphenous lymphatic vessels with saphenous veins in rats.[14,15]

Our previous investigations show that rabbits are ideal candidates for inducing secondary lymphedema and performing surgical procedures, including popliteal lymph node excision.[16,17] The distinctive anatomical attributes of the rabbit hindlimb render it amenable to the faithful reproduction of surgical techniques practiced in human patients.[18] Consequently, rabbits are frequently used as models for microsurgical training and preclinical investigations, thereby facilitating the extrapolation of research findings to human medicine.[19,20] This study aimed to implement an experimental methodology for the surgical application of the LVA technique using lymphatic vessels and popliteal veins located in the popliteal region of rabbit hindlimbs. This methodology aims to establish a training platform for surgeons in the field of supermicrosurgery.

Materials and methods

After obtaining the approval from the institutional review board, this study was conducted at the Jesús Usón Minimally Invasive Surgery Center (Cáceres, Spain). All parameters were assessed according to guidelines for quantifying pain, stress, and distress in laboratory animals.[21-24]

This investigation was designed to assess and implement a supermicrosurgical training methodology for LVA in an animal model. The study was conducted using a cohort of 10 healthy 1-year-old male rabbits (New Zealand White rabbits; Granja San Bernardo, Tulebras, Navarra, Spain), and the surgical procedures were performed on both hindlimbs. The study population was randomly divided into two groups using Microsoft Excel® Microsoft 365 MSO (Version 2207 Build 16.0.15427.20248), each comprising 5 animals, resulting in 10 LVAs within each group. Indocyanine green (ICG) lymphography was used to establish the anastomotic site and evaluate the patency of the LVA within the popliteal fossa of both hindlimbs. For each animal, two digital video cameras equipped with an infrared filter (Pentero 800 s [ZEISS, Jena, Germany] and Fluobeam [Fluoptics, Grenoble, France]) were used to capture and record the ICG contrast (25 mg; Verdye; Diagnostic Green Limited, Westmeath, Ireland; Figure 1). Patent Blue V (PBV) (2.5 g/100 mL; Bleu Patente V Sodique; Guerbet, Villepinte, France) dye was used to visualize the lymphatic vessels intraoperatively in the anastomosis zone. Supermicrosurgical instruments were used for all surgical procedures (EMI Set A-supermicro; Mitaka Europe Gmbh, Kurfürstendamm, Berlin, Germany). Follow-up, euthanasia, and sample collection procedures were performed at 15 and 30-days post-surgery. Two independent researchers collected the data.

The co-induction phase of anesthesia was initiated by administering midazolam (5 mg/mL; Normon SA, Madrid, Spain) at a dose of 0.5 mg/kg and propofol (10 mg/mL; Propomitor;

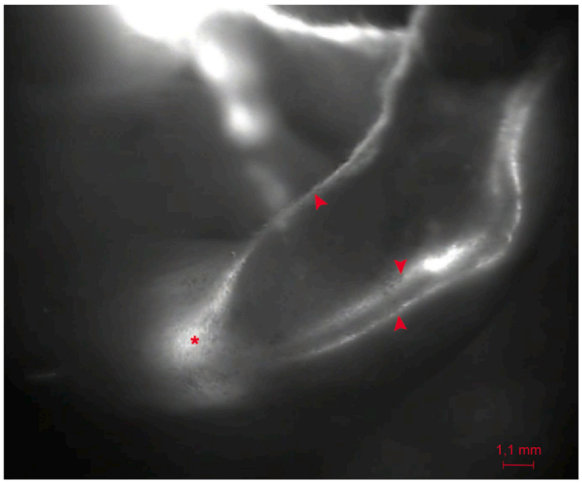

Figure 1 Preoperative findings in a rabbit model before lymphaticovenous anastomosis. Viability of the popliteal lymph node (red asterisks) and hindlimb lymphatic vessels (red arrowheads) was confirmed using ICG lymphography. ICG, indocyanine green.

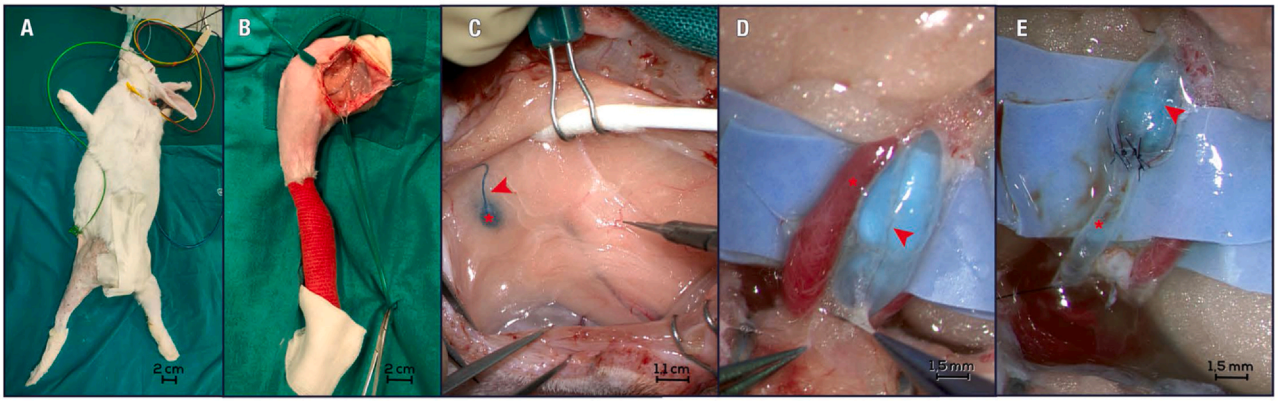

Figure 2 A, Positioning and general anesthesia of the rabbit with the left hindlimb shaved preoperatively. B, Surgical approach in the popliteal area. C, Identification of the popliteal lymph node (red arrowheads) using PBV. D, Popliteal vein (red asterisks) and afferent lymphatic vessel (red arrowheads) identification using PBV. E, Lymphaticovenous anastomosis was performed between the afferent lymphatic vessel (red arrowheads) and popliteal vein (red asterisks) using the end-to-end technique with single stitches. Patency was assessed using PBV. PBV, Patent Blue V.

Orion Pharma, Spoo, Finland) at a dose of 10 mg/kg via infusion into the ear marginal vein. All rabbits were subsequently intubated using 3.0–3.5 endotracheal tubes connected to a semi-closed circuit, maintaining sevoflurane (1000 mg/g; SevoFlo; Zoetis Belgium, Luvain-la-Neuve, Belgium) at a concentration of 3.9–4.5%. During the intraoperative phase, analgesia was achieved by administering ketorolac (30 mg/mL; Normon SA) at a dose of 1.5 mg/kg and tramadol (50 mg/mL; Normon SA) at a dose of 3 mg/kg.

In the post-operative period, buprenorphine (300 µg/mL; Bupaq; Richter Pharma, Wels, Austria) was administered at a dose of 30 mg/kg, along with meloxicam (5 mg/mL; Meloxidyl; Ceva Santé Animale, Libourne, France) at a dose of 2 mg/kg and enrofloxacin (10 mg/mL; Baytril; Bayer Animal Health Gmbh, Leverkusen, Germany) at a dose of 5 mg/kg, and continued for 5 days post-surgery. An anesthetic protocol identical to that described previously was employed during the 15- and 30-day post-operative follow-up assessments. Euthanasia was performed using intravenous potassium chloride (20 mmol/10 mL; B. Braun Medical SA, Barcelona, Spain) at an average rate of 2 mEq/kg into the auricular vein.

Before surgery, the study subjects were placed in the prone position, and meticulous depilation of their hindlimbs up to the groin region was performed, followed by skin antisepsis. To visualize the vessels and lymph node, we administered 0.2 mL of PBV intradermally into the second and third interdigital spaces of both hindlimbs. After injection, the area was gently massaged, and controlled flexion and extension movements of the hindlimbs were performed for a few minutes to facilitate dye absorption into the lymphatic vessels. Open surgery was performed in the popliteal fossa to identify the popliteal vein and afferent lymphatic vessels leading to the popliteal lymph nodes. These two vascular structures were meticulously dissected from the arterial and collateral venous networks, and their diameters were measured using a surgical ruler. The distal end of the lymphatic vessel was ligated to prevent unintentional leakage of ICG and PBV during subsequent surgical assessments. A comparable precaution was applied to the proximal end of the vein to ensure readiness for anastomosis.

The lymphatic vessel and popliteal veins were cut, and LVAs were performed under a surgical microscope, facilitating the unidirectional flow of lymph from the distal lymphatic vessel to proximal popliteal vein. End-to-end anastomosis was meticulously performed using six or eight 11–0 monofilament nylon non-absorbable sutures (Covidien; Mansfield, MA, USA; Figure 2). ICG lymphography and PBV were used to evaluate the lymphatic fluid flow from the lymphatic vessel to the popliteal vein and to confirm the patency of the anastomosis. The skin incision was sutured using 4-0 polyglycolic acid absorbable sutures (Aragó; Barcelona, Spain) in a continuous intradermal pattern and a single-stitch technique to mitigate the risk of post-operative self-mutilation.

Follow-up examinations were conducted at 15- and 30-days post-operatively. A longitudinal incision was made at the same anatomical site as in the initial surgical procedure, enabling the assessment of anastomotic patency via ICG lymphography and PBV (Figures 3 and 4). Following data acquisition, samples of the LVAs were harvested for subsequent histopathological analysis, and the subjects were euthanized using ethical procedures.

Hematoxylin and eosin staining was used to histologically examine the region approximately 0.5 cm from the anastomotic site. Histological sections were digitally scanned using the Aperio GT 450 DX system (Leica Biosystems, Barcelona, Spain). The assessed parameters included endothelial loss, thrombosis, superficial fibrin deposition, presence of fibrin within the neointima or media, calcification, semi-quantitative appraisal of neointimal thickness at the anastomotic regions, neoangiogenesis (evaluated semi-quantitatively), and the extent and distribution of inflammation.

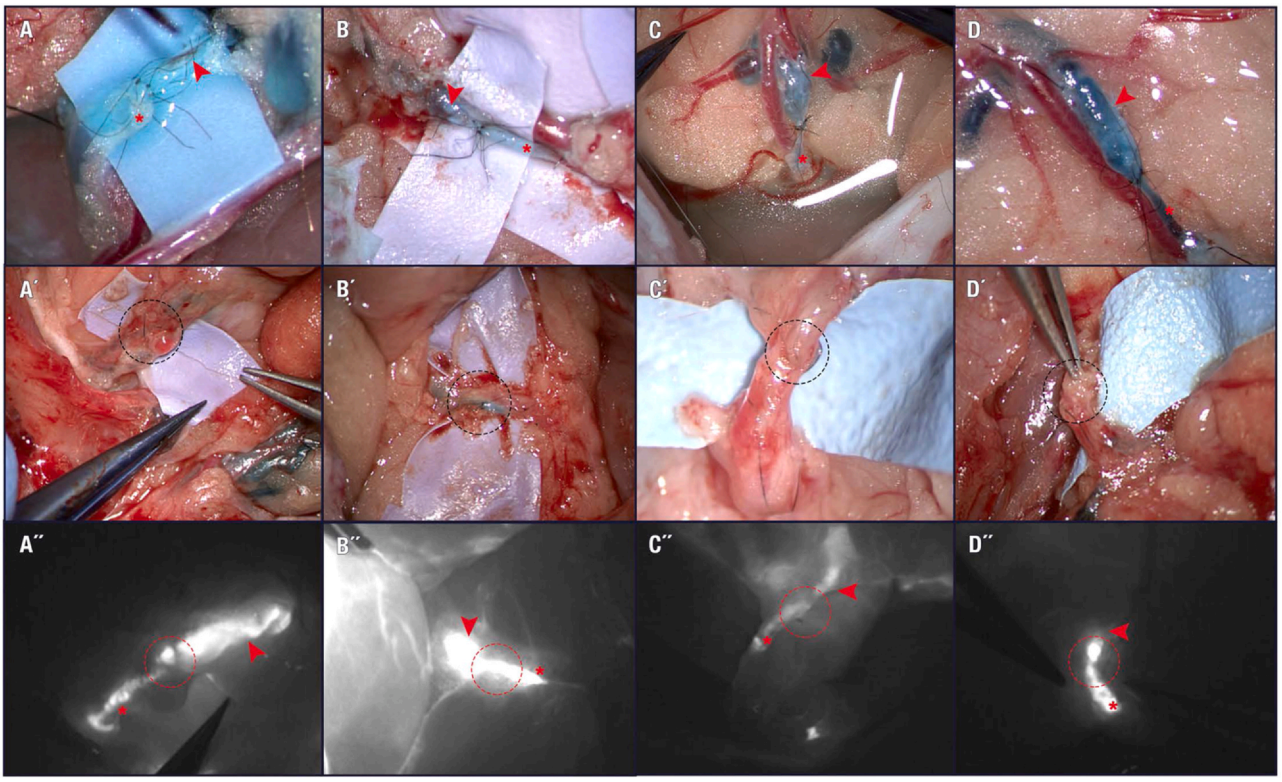

Figure 3 Follow-up and anastomotic patency assessment at 15 days post-surgery using ICG and PBV. A, Patent lymphaticovenous anastomosis confirmed using PBV on day 0. The afferent lymphatic vessel (red arrowheads) and popliteal vein (red asterisks) are visible. A', Lymphaticovenous anastomoses observed under a surgical microscope 15 days post-surgery. Scar tissue surrounding the anastomotic site is evident (black circle). A", Assessment of anastomotic patency using ICG (red circle). Lymphatic fluid is observed flowing from the lymphatic vessel (red arrowheads) into the vein (red asterisks). ICG, indocyanine green; PBV, Patent Blue V.

Results

Ten rabbits were divided into two groups and subjected to supermicrosurgical LVA technique in both hindlimbs, totaling 20 LVAs. The initial group, comprising 5 rabbits, underwent euthanasia 15 days post-surgery. The second group, comprising 5 rabbits, was euthanized after 30-days.

In the described supermicrosurgical procedure, following the execution of the LVA technique, there were no apparent impairments in hindlimb blood perfusion, no deviations in vital physiological parameters recorded under anesthesia, and no pain or lameness.

The mean weight of the rabbits was 5.3 (range, 4.5–6.0) kg. The mean diameters of the 20 lymphatic vessels and veins were 1.0 (range, 0.6–1.5) mm and 0.8 (range, 0.3–2.0) mm, respectively. The median number of collateral veins within the primary vein was 3 (range, 0–6). The initial postoperative patency rate for the LVAs was 90% (19/20 patients). The patency rates 15 and 30-days after surgery were 60% (6/10) and 80% (8/10), respectively. The mean time from the initial cutaneous incision to the evaluation of LVA patency was 65.8 (range, 45–180) min (Table 1).

During both follow-up evaluations, we observed the progressive formation of fibrotic tissue encircling the anastomotic sites, rendering visual inspection of the sutures challenging. One of the subjects engaged in self-mutilation at the incision site, resulting in the unavailability of LVA 15 days post-surgery.

Histological analysis

Mild endothelial loss was evident in 3 cases, without any discernible presence of fibrin in the de-endothelialized regions (Figure 5). Thrombosis or superficial fibrin deposition was observed in a single case (Figure 6). None of the samples exhibited calcification, and the neointimal thickness was less than that of the medial wall. Neoangiogenesis was uniform in all samples, displaying varying degrees of intensity, generally ranging between 6 and 10 neoformed vascular structures (Figure 7). Signs of inflammation ranging from mild to moderate were observed in all cases. In addition, lymphatic valves were present, and the structure of the lymphatic ducts closely approximated normalcy.

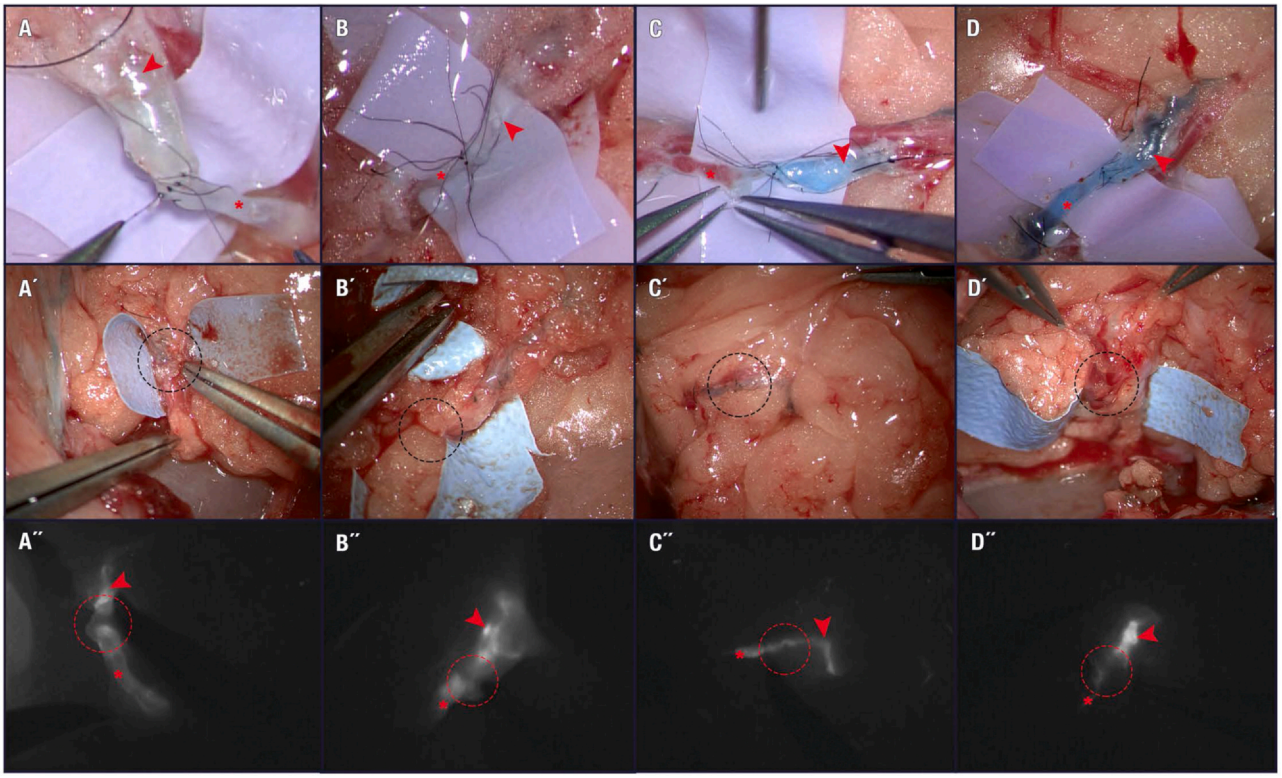

Figure 4 Follow-up and anastomotic patency assessment at 30-days post-surgery using ICG and PBV. A, Patent lymphaticovenous anastomosis confirmed using PBV on day 0. The afferent lymphatic vessel (red arrowheads) and popliteal vein (red asterisks) are visible. A', Lymphaticovenous anastomoses observed under surgical microscope 30-days post-surgery. Scar tissue surrounding the anastomotic site is evident (black circle). A", Assessment of anastomotic patency using ICG (red circle). Lymphatic fluid is observed flowing from the lymphatic vessel (red arrowheads) into the popliteal vein (red asterisks). ICG, indocyanine green; PBV, Patent Blue V.

Discussion

Attaining proficiency in microsurgery is imperative to achieving expertise as a skilled microsurgeon. Numerous publications have addressed microsurgery training, providing valuable insights and strategies for improving technical skills in this specialized field.[25] Conventional training programs focus on acquiring technical microsurgical skills by employing silicone tubes and animal models, including chicken vessels and rat femoral arteries or veins.[26-28] These models provide valuable hands-on experience and are effective tools for honing microsurgical techniques and developing the dexterity and precision required in this demanding field.

The diameter of the lymphatic vessels involved in LVA procedures is usually < 1 mm, requiring advanced skills compared to those required for conventional microvascular anastomosis.[29] Supermicrosurgery of lymphatic vessels presents distinct and challenging characteristics compared to microsurgery.[30] Transparent lymphatic vessels pose difficulties in defining the boundary between the adventitia and surrounding connective tissue.[31] Additionally, the soft and delicate nature of lymphatic ducts makes their management challenging, necessitating the adoption of an atraumatic surgical technique.[32]

Using the LVA surgical technique in live experimental animal models is a novel approach with the potential to provide surgical training for surgeons. Advancements in this technique have the potential to establish a preclinical model, aiding in the prevention and treatment of pathophysiological processes such as lymphedema.[33]

Various authors have documented the use of animal models to conduct LVA, specifically in dogs,[8,9] pigs,[10] and rats.[11-15] Despite instances in which the diameters of lymphatic vessels and veins in these animal models resemble those in humans,[11] there are several drawbacks in their management and housing. Among these, rat models have been extensively investigated. Rat models are affordable and easily housed; however, any surgical intervention performed on these small experimental animals carries inherent risks to their survival, attributable to the surgical procedure and anesthesia. Furthermore, there is a potential risk of self-mutilation at the surgical site and challenges associated with post-operative monitoring.

Table 1 Description of the experimental rabbit lymphaticovenous anastomosis model.

Animal number	Right hindlimb lymphatic vessel (mm)	Left hindlimb lymphatic vessel (mm)	Right hindlimb vein (mm)	Left hindlimb vein (mm)	Right anastomotic patency (0/15/30)	Left anastomotic patency (0/15/30)
1	1.5	1.5	0.5	2	⊘/⊖/×	⊖/⊖/×
2	1	2	0.5	1.5	⊘/⊖/×	⊘/⊘/×
3	1.2	1.5	0.8	0.9	⊘/⊘/×	⊘/⊘/×
4	1	1.2	0.8	1	⊘/⊘/×	⊘/⊖/×
5	1	1.1	1.5	0.5	⊘/⊘/	⊘/⊘/×
6	0.6	0.6	0.9	0.5	⊘/× /⊖	⊘/× /⊘
7	0.7	1	0.6	1.2	⊘/× /⊘	⊘/× /⊖
8	0.9	0.9	0.3	0.3	⊘/× /⊘	⊘/× /⊘
9	0.7	0.7	0.3	0.9	⊘/× /⊘	⊘/× /⊘
10	1	0.9	0.6	0.3	⊘/× /⊘	⊘/× /⊘
Main	1.0		0.8		90/60/80 (%)	

LVA, lymphaticovenous anastomosis; 0, immediately after LVA; 15, 15 days later; 30, one month later; ⊘, permeable LVA; ⊖, non-permeable LVA; ×, no follow-up.

We believe that the use of rabbits as training animal models surpasses other options because of their optimal average size for handling and housing.[34] Moreover, rabbits possess anatomical characteristics that allow the replication of surgical techniques performed in humans.[16,18] In particular, despite the presence of distinct superficial afferent lymphatic vessels in the right and left hindlimbs of rabbits, the deep vascular bundle within the popliteal fossa, comprising the popliteal artery, popliteal vein, and afferent lymphatic vessels, is a favorable site for LVA. This site features larger lymphatic vessels and offers convenient access upon excision the subcutaneous fat, without the dissection of the adjacent muscle tissue. Furthermore, there are no major vascular structures nearby at risk of injury, thereby avoiding potential risks to the animal's life. Additionally, post-operative follow-ups can be conducted, as these procedures pose minimal risk to the rabbits' lives and exhibit remarkable healing capacity.

The mean diameter of the lymphatic vessels and popliteal veins in this particular model was 1.0 mm and 0.8 mm, respectively. When comparing these parameters with other animal models, the mean diameter of lymphatic vessels in rats ranges from 0.61 mm to 0.24 mm.[11-15] In comparison, it was approximately 1.0 mm in pigs.[10] Unfortunately, no data is available regarding the diameters of the lymphatic vessels and veins in dogs.[8,9] Regarding the veins, our observations indicate that the mean diameter in rats ranges from 0.37 mm to 0.81 mm.[11-15] Regrettably, there is a dearth of data on vein diameters in pigs. In comparison, our animal model exhibited a larger lymphatic vessel/venous size than the previously mentioned animal models, facilitating vascular dissection and rendering it more manageable even for inexperienced surgeons. This provides a significant opportunity for refining skills in dissecting lymphatic vessels, veins, and arteries.

During the surgical procedure, PBV was used to assess the presence or absence of flow within the hindlimb lymphatic vessels.[35] However, this technique has limitations because it can only be performed intraoperatively. To overcome this limitation, we used real-time near-infrared fluorescence imaging technology, a superior method for identifying, mapping, and quantifying lymphatic flow in the lymphatic channels at the ICG application site without requiring invasive surgical intervention.[36] This innovative approach enables real-time visualization of lymphatic dynamics and provides valuable insights for research and clinical applications.

Following the anastomosis, a patency test was conducted to evaluate the presence of countercurrent lymph flow in the LVA region, thereby determining the success or failure of the procedure. Subsequently, the patency of the anastomosis was confirmed using ICG lymphography. Furthermore, the use of PBV enabled direct visual observation of the flow of the dye from the lymphatic vessel to popliteal vein. Regarding the duration of the anastomosis procedure, the average time from the first incision to the patency evaluation was 65.8 min, which is consistent with the findings of earlier investigations.[11,12,15]

We observed a smooth endothelial transition without exposure of the subendothelial tissue layers in cases where permeability was achieved. Conversely, in the three impermeable samples, endothelial loss was observed without

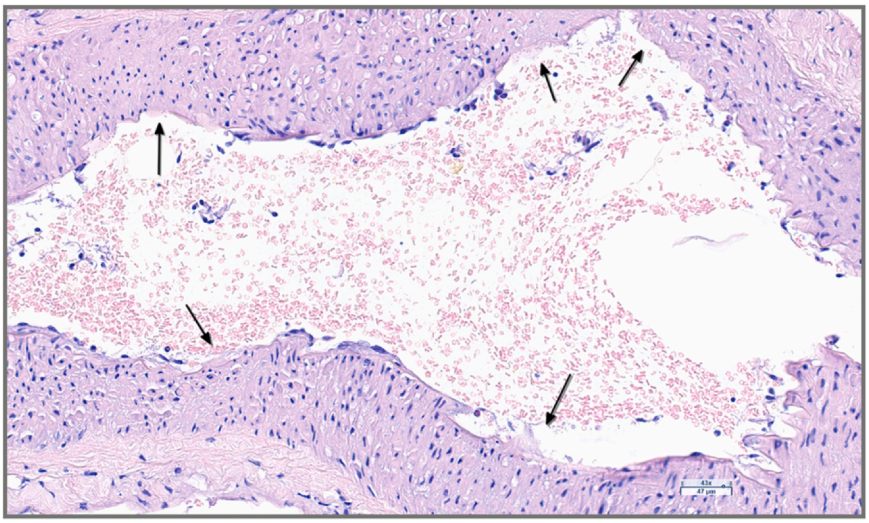

Figure 5 Cross-sections of the lymphatic vessel in a patent case (hematoxylin and eosin staining) reveal a mild area of endothelial loss (black arrowheads).

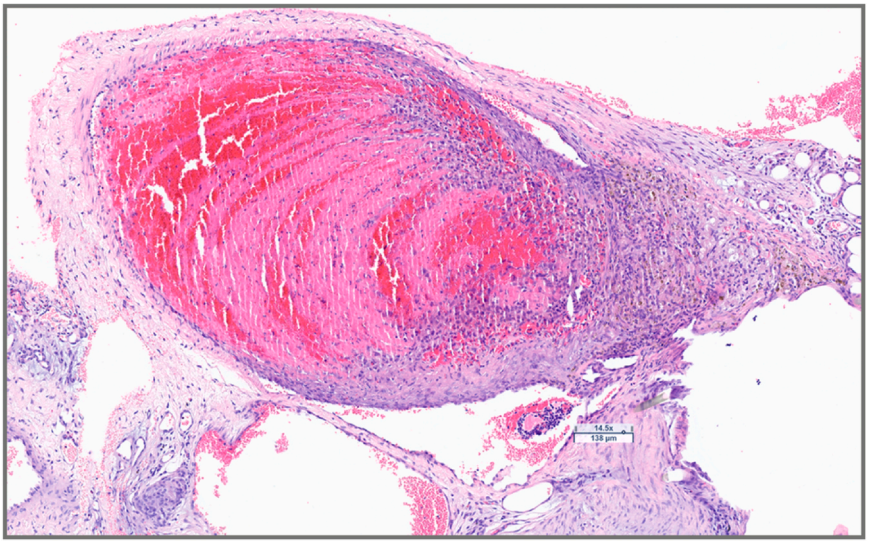

Figure 6 Cross-sections of the lymphatic vessel (hematoxylin and eosin staining) show vascular thrombosis with associated inflammation.

fibrin. Thrombosis and fibrin in the medial or neointimal layer were identified in only one non-permeable sample. Simultaneously, inflammation was observed in all cases, primarily due to the local suture thread reaction. Collectively, these factors appear to be significant contributors to long-term patency.

Our findings demonstrated favorable lymphatic patency in the hindlimbs of rabbits throughout the study period following the implementation of LVA procedures. These results indicate the promising establishment of an experimental animal model suitable for training purposes.

This study had some limitations. This technique requires live animals and its use should be minimized by ethical considerations.[37] From an ethical standpoint, employing this model during the advanced stages of training after practice with ex vivo supermicrosurgery models or inert materials is preferable.[26,38–40] Another limitation inherent to this model is the variability in the observed diameters of veins and lymphatic vessels; notably, in some instances, the afferent lymphatic vessel exhibits a larger diameter than the popliteal vein, thereby introducing heightened complexity into the surgical procedure.

In conclusion, this study provides reasonable evidence that rabbits are reliable and reproducible experimental animal models for performing LVA. The anatomy of the lymphatic system of the popliteal area of the hindlimb enables the practice of supermicrosurgical techniques and facilitates improvement of dissection skills owing to the comparatively larger size of vascular structures compared to alternative animal models. Furthermore, it provides a

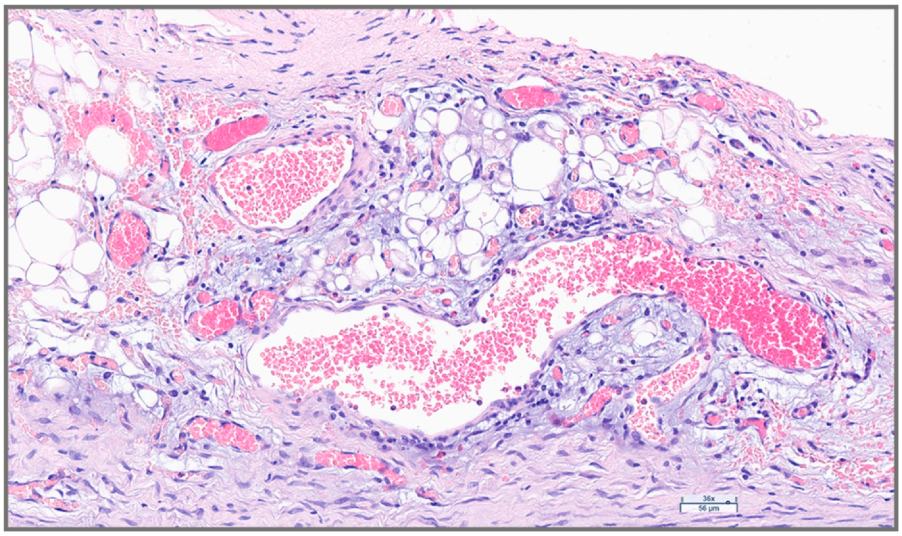

Figure 7 Digital microscopic image (Aperio GT 450 DX system) displaying neoangiogenesis in all cases, typically with 6–10 newly formed vascular structures.

surgical field readily accessible by excising the subcutaneous fat layer without necessitating entry into the musculature of the hindlimb.

Ethics

All experimental procedures conducted in this study were approved by the Ethics Committee of the Jesús Usón Minimally Invasive Surgery Center (Cáceres, Spain) and the Government of the Junta de Extremadura (EXP-20231219), in accordance with the European legislation.

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Conflict of interest statement

None.

Previous presentations

None.

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