Title Page

Title: **Microsurgical Cervical Lymphaticovenous Anastomosis: A Novel Mouse Model for Brain Lymphatic Outflow**

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Abstract

Background:

Cervical lymphaticovenous anastomosis (LVA) has recently emerged as an innovative surgical approach to enhance brain lymphatic clearance for the treatment of Alzheimer's disease (AD). While early clinical findings are promising, the absence of a reproducible preclinical model has hindered mechanistic investigations and translational advancements.

Objective:

This study aimed to establish the first mouse model for cervical LVA, addressing technical challenges and providing a standardized platform for preclinical research on lymphatic clearance and its role in neurodegeneration.

Methods:

Mice underwent bilateral cervical LVA using high-magnification supermicrosurgical techniques. Following identification of the deep cervical lymph nodes, end-to-side anastomosis was performed between the deep cervical lymphatic vessels and the external jugular vein. Anastomotic patency was confirmed intraoperatively by direct visualization without leakage. Indocyanine green (ICG) lymphangiography, performed via nasal administration, demonstrated fluorescence drainage into the

external jugular vein.

Results:

With progressive refinement of microsurgical skills and improved anatomical familiarity, procedural stability was achieved. In the final cohort of five mice, all LVA procedures resulted in successful anastomotic patency. Intraoperative indocyanine green (ICG) lymphangiography confirmed real-time lymphatic drainage into the venous system in all cases.

Conclusion:

This study demonstrates that lymph node–venous anastomosis (LNVA) is a feasible approach for cervical lymphaticovenous anastomosis (LVA) in mice, overcoming previous technical limitations. As LNVA is also the predominant LVA technique now used in Alzheimer’s disease patients in China, this model provides a valuable platform for investigating its role in brain lymphatic drainage and neurodegeneration. Additionally, we propose nasal ICG lymphangiography as an effective imaging method for assessing lymphatic flow, offering a more effective alternative to local injection. This model lays the groundwork for future studies on LNVA’s impact on glymphatic clearance and potential clinical applications.

Keywords

Cervical lymphaticovenous anastomosis, Glymphatic System, Alzheimer's Disease, microsurgery, Indocyanine Green, Animal Model.

Introduction

Lymphaticovenous anastomosis (LVA) is a minimally invasive supermicrosurgical technique used to restore lymphatic drainage by connecting lymphatic vessels, typically less than 0.8 mm in diameter.(1)This procedure has demonstrated efficacy in managing extremity lymphedema, with a growing body of evidence supporting its application in peripheral lymphatic disorders. More recently, Prof. Xie has applied this technique to Alzheimer's disease (AD) patients, garnering widespread attention.(2,3)

Alzheimer's disease (AD) is the most common cause of dementia, affecting over 35 million people globally, with prevalence expected to reach 115 million by 2050 due to population aging.(4) Although the recently developed lecanemab and Donanemab have shown some promise in slowing early-stage disease progression,(5,6) there remain no effective treatments capable of reversing or significantly delaying Alzheimer's progression, particularly in moderate-to-severe cases.(7) Historically, the central nervous system (CNS) was considered devoid of lymphatic structures. This understanding shifted dramatically in 2015 with the discovery of lymphatic vessels within the meninges, revealing a previously unrecognized central nervous lymphatic system.(8) This discovery has redefined our understanding of CNS physiology and its links to neurodegenerative diseases, particularly Alzheimer's disease (AD). Impaired glymphatic clearance, the brain's process for removing metabolic waste such as amyloid-beta, is now recognized as a key contributor to AD pathology.(9) Efforts to enhance glymphatic clearance have since emerged as a promising therapeutic strategy

with preclinical studies demonstrating encouraging results using AQP4 channel modulation,(10) multi-sensory gamma stimulation(11), and focused ultrasound.(12)In China, through Xie et al.'s efforts in sharing and advancing this technique, LVA has rapidly gained adoption as a surgical approach for Alzheimer's disease. To date, over 100 hospitals have initiated LVA procedures for AD, with many institutions registering clinical trials to evaluate its therapeutic potential(ChiCTR2400095878;ChiCTR2400092047;ChiCTR2400089883;ChiCTR2400084671;ChiCTR2500095309;ChiCTR2400091857;ChiCTR2500099251). This widespread clinical adoption highlights the pressing need for a standardized preclinical model to systematically study its mechanisms and refine surgical protocols.To address this gap, we have optimized microsurgical techniques, refined surgical protocols, and developed an effective intraoperative ICG imaging strategy. By establishing this animal model, we aim to advance cervical LVA research and provide a platform for further investigation into its therapeutic potential.

Methods

Animal Model and Ethics Approval

All procedures were approved by the Zhejiang Province Hospital Animal Care and Use Committee (IACUC) (Approval Number: 20241105094790) and conducted in accordance with the China National Guide for the Care and Use of Laboratory Animals. Adult C57BL/6J mice (8–12 weeks old, 22–28 g, male) were obtained from Hangzhou Qizhen Laboratory Technique Company and housed under standard

laboratory conditions (temperature: 22±2°C, humidity: 50±10%, 12-hour light/dark cycle) with ad libitum access to food and water. Mice were acclimatized for seven days prior to surgery to minimize stress and ensure physiological stability.

Surgical Procedure

Mice were anesthetized via intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Anesthesia depth was monitored by assessing the absence of the pedal withdrawal reflex and maintaining a stable respiratory rate. To prevent corneal desiccation, ophthalmic ointment was applied to the eyes, and body temperature was maintained at 37°C using a heated surgical platform.

A 1.5–2.0 cm midline cervical incision was made under sterile conditions(Figure 1A), and the subcutaneous tissue and platysma muscle were carefully retracted using fine microsurgical instruments to expose the deep cervical lymphatic structures.

The external jugular vein was meticulously dissected and mobilized under high-magnification microscopy (20–40×) to avoid vascular injury(Figure 1B). Adjacent to the vein, the deep cervical lymph nodes (DCLNs) were identified and carefully separated from surrounding connective tissue(Figure 1C). To facilitate anastomosis, a portion of the deep cervical lymph node was excised, optimizing its positioning for lymphatic-venous connection. The external jugular vein was temporarily occluded using bilateral microvascular clamps, with a scale and support pad placed to enhance microsurgical precision(Figure 1D,1E) .

A delicate end-to-side anastomosis was performed between the efferent lymphatic from the deep cervical lymph node and the external jugular vein using 11-0 nylon sutures(Figure 1F). Upon gradual release of the vascular clamps, real-time intraoperative assessment confirmed anastomotic patency, with no visible leakage observed upon gross examination.

Indocyanine Green (ICG) Lymphangiography

To assess lymphatic-venous connectivity, 0.05 mL of 0.25 mg/mL ICG was administered via unilateral nasal instillation, targeting the nasal mucosa. The injection was performed as superficially as possible, stopping upon sensing slight resistance to avoid deeper penetration and unintended vascular entry. A 30-minute waiting period was observed to allow for optimal ICG distribution within the lymphatic system before imaging.

Results

With progressive refinement of microsurgical techniques and improved anatomical familiarity, procedural stability was achieved in performing cervical lymphaticovenous anastomosis (LVA) in mice. In the final cohort of five mice, all procedures were successfully completed, with anastomotic patency confirmed intraoperatively. Upon gradual release of microvascular clamps, the anastomotic site

demonstrated continuous flow without leakage, indicating successful lymphatic-to-venous drainage.

To validate the functionality of the anastomosis, indocyanine green (ICG) lymphangiography was performed using unilateral nasal instillation (0.05 mL, 0.25 mg/mL), targeting the nasal mucosa. Compared to traditional local injection, nasal administration provided superior lymphatic visualization with minimal background fluorescence and contamination. Fluorescence imaging revealed clear drainage of ICG into the external jugular vein, confirming successful lymphatic-venous connectivity in all five mice(Figure 1G,1H).

Postoperatively, all mice recovered without immediate complications. After confirmation of stable vital signs, humane euthanasia was performed to prevent prolonged postoperative discomfort

Discussion

As a commonly used microsurgical lymphatic anastomosis technique, LVA has been explored across disciplines for the treatment of neurological disorders such as Alzheimer's disease.(2,13) However, high-level clinical evidence and experimental validation remain lacking, making the development of an animal model to evaluate this procedure highly necessary, yet its underlying mechanisms remain incompletely understood. Animal studies have shown that bilateral ligation of deep cervical lymph

nodes accelerates AD pathology(14), while pharmacological strategies to enhance cervical lymphatic flow improve AD disease outcomes in mouse models.(15) In patients with head and neck cancer, bilateral and extensive cervical lymph node dissection is associated with an increased risk of dementia, further highlighting the critical role of cervical lymphatic drainage in brain health.(16) Clinically, deep cervical lymph nodes from AD patients have been found to contain significant amyloid-β (Aβ) deposition and inflammatory infiltration.(17,18) During our clinical surgery, we consistently noted enlarged and reactive lymph nodes, further reinforcing the idea that the deep cervical lymph nodes are a key site of pathology in AD. These findings suggest that the cervical lymphatic system may serve as a crucial therapeutic target for neurological diseases.

The therapeutic effects of cervical LVA remain incompletely understood. One hypothesis is that it bypasses dysfunctional lymph nodes, restoring normal lymphatic drainage. Another is the “vis a fronte” effect,(19) where the jugular vein’s to the right atrium creates a pressure gradient driven by cardiac pulsation and respiration, facilitating the movement of lymphatic fluid into the venous system like a pump. Interestingly, emerging evidence suggests that lymphedema is not merely a peripheral condition but also has implications for central nervous system function. Studies have reported that patients with limb lymphedema exhibit a threefold higher incidence of cognitive impairment compared to controls, and that surgical intervention to alleviate limb lymphedema can lead to metabolic improvements. These observations underscore the intricate relationship between the lymphatic system and brain function,

highlighting the need for further investigation into its broader neurological implications.(20) While both mechanisms are plausible, they remain unproven. In our future research, we will focus on further exploring these mechanisms to deepen our understanding of their impact.

Current AD animal models are primarily based on transgenic mice, such as Tg2576, 3xTg-AD, and P301L-Tau models, which replicate key pathological features including Aβ plaque deposition, tau hyperphosphorylation, and cognitive deficits, though they do not fully capture the complexity of human AD.(21) Although mice were previously considered unsuitable for cervical lymphaticovenous anastomosis due to their small size, we successfully established this model and validated it with ICG imaging through meticulous refinement and procedural optimization. Here, we share our insights and technical considerations for the surgical modeling process. The cervical anatomy of mice presents significant microsurgical challenges, requiring extreme precision due to the small caliber of lymphatic and venous structures. The superficial cervical lymph nodes are encountered first and can be easily mistaken for deep cervical lymph nodes, necessitating careful identification. A sternocleidomastoid muscle, similar to that in humans, is also present, with deep cervical lymph nodes located beneath it and adjacent to the internal jugular vein. Previous studies report an average of 6 deep cervical lymph nodes in humans,(22) While preparing cervical lymph node transfer flaps in rats, researchers identified 4–6 lymph nodes in the cervical region.(23) Similarly, during our surgical procedures, we also observed a chain of approximately 4–6 deep cervical lymph nodes in mice. Given that the

external jugular vein is larger than the internal jugular vein in mice, we opted for an end-to-side anastomosis to ensure patency and minimize the risk of postoperative stenosis. Meticulous handling is essential to prevent lymphatic vessel damage, and we ultimately selected the deep cervical lymph node closest to the skull base for anastomosis.

ICG lymphangiography is essential for verifying postoperative patency. Initially, we used local injection, but this often resulted in field contamination, limiting the clarity of imaging. Exploring alternative approaches, we were influenced by reports highlighting the nasal cavity as a key lymphatic drainage pathway.(24) By administering ICG via unilateral intranasal injection and waiting approximately 30 minutes, we observed clear fluorescence within the cervical lymphatic system, with subsequent drainage into the venous system through the anastomotic site. This not only supports the nasal route as a crucial lymphatic drainage pathway but also suggests that intranasal injection allows retrograde flow into the brain before reaching the deep cervical lymph nodes. To minimize interference, the injection should be kept superficial within the nasal mucosa, avoiding deeper layers or direct vascular entry, which could cause imaging artifacts from venous contamination. This method may serve as a valuable technique for studying brain lymphatic circulation and could provide a standardized approach for future investigations into glymphatic-lymphatic connections.

Due to ethical constraints, this study focused on intraoperative patency validation without long-term postoperative assessment. Further studies are needed to evaluate

long-term anastomotic stability and its functional impact on glymphatic clearance. Moving forward, we aim to collaborate with neurology and basic research teams to explore the broader implications of this model in neurodegenerative disease. Additionally, refinements in surgical techniques remain an open area for optimization, including the potential for multi-site anastomoses involving lymph nodes closer to the skull base.In the mouse model, lymphaticovenous anastomosis (LVA) at the lymphatic vessel level proved technically unfeasible due to the extreme vessel size, leading us to perform lymph node–venous anastomosis (LNVA) instead. This technique is currently the predominant surgical approach used in China for LVA in Alzheimer's disease patients.

Conclusion

This study establishes the first reproducible mouse model of cervical LNVA, demonstrating its feasibility and technical reproducibility. Given that LNVA is currently the predominant surgical approach used for LVA in AD patients in China, this model serves as a critical platform for investigating its impact on brain lymphatic drainage and neurodegenerative pathology. Additionally, we introduce nasal ICG lymphangiography as a superior imaging method.

Moving forward, this model will facilitate studies evaluating the impact of LNVA on glymphatic clearance, Aβ metabolism, neuroinflammation, bridging fundamental research with clinical applications in neurodegenerative disease management.

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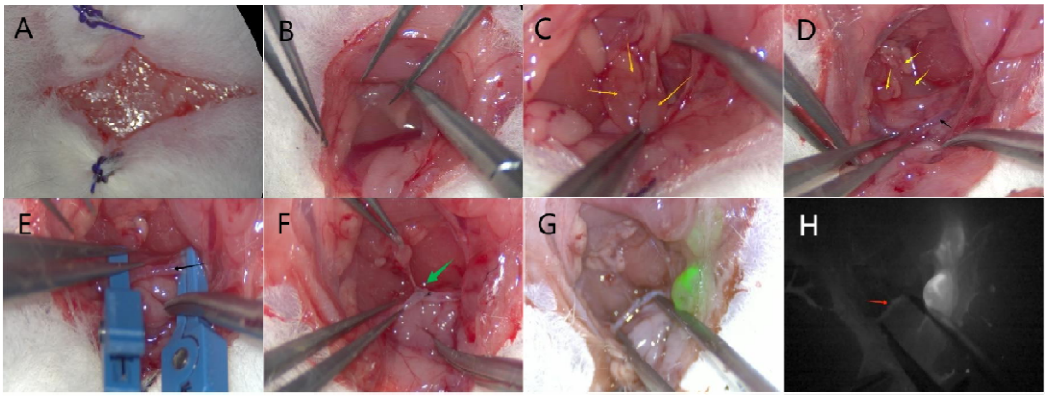


Figure 1. Stepwise procedure of cervical lymphaticovenous anastomosis (LVA) in the mouse model.
1A. Midline cervical incision in a mouse.
1B. Microsurgical approach to the cervical region.
1C. Deep cervical lymph nodeslocated beneath the sternocleidomastoid muscle
1D. The external jugular vein is carefully dissected and exposed (black arrow).
1E. Prepare the external jugular vein (black arrow)
1F. End-to-side anastomosis is completed using 11-0 sutures. The green arrow indicates the anastomotic site, where the lymphatic vessels connect to the external jugular vein.
1G. Postoperative indocyanine green (ICG) lymphangiography.
1H. The red arrow marks the anastomotic site, where ICG fluorescence is visible .

Conflict of Interest Statement

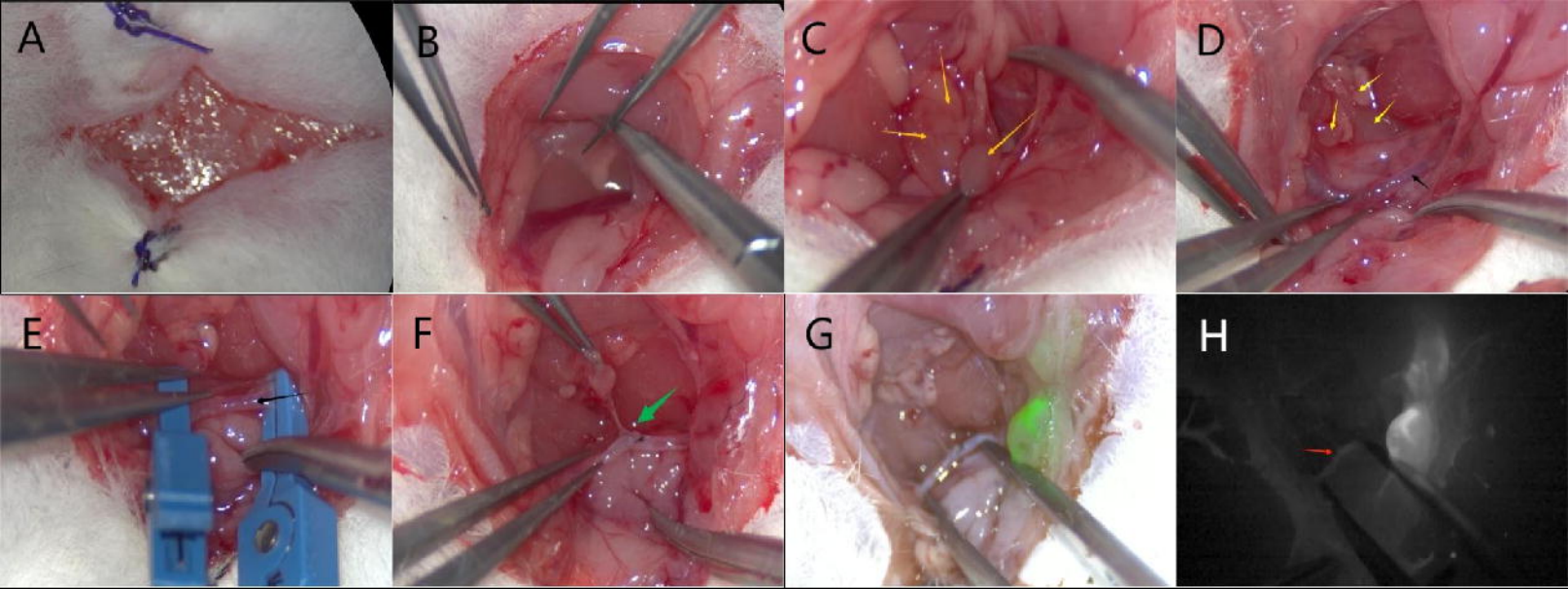
The authors declare no conflicts of interest related to this work.

Data Availability Statement

The data supporting this study are available upon reasonable request from the corresponding author, subject to institutional policies.

Declaration of Generative AI and AI-Assisted Technologies

While preparing this work, the authors, as non-native English speakers, used ChatGPT (OpenAI) to enhance language clarity and readability. After utilizing this tool, the authors carefully reviewed and edited the content to ensure accuracy, coherence, and alignment with the research objectives. The authors take full responsibility for the final content of this publication.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