Received: 26 December 2023 | Accepted: 26 February 2024

DOI: 10.1002/rmb2.12570

Reproductive Medicine and Biology

WILEY

ORIGINAL ARTICLE

Novel erectile analyses revealed augmentable penile Lyve-1, the lymphatic marker, expression

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Funding information
the Japan Society for the Promotion of Science grants, Grant/Award Number: 21K06822; Grant-in-Aid for Japan Society for the Promotion of Science Fellows, Grant/Award Number: 23KJ1859

Abstract

Purpose: The pathophysiology of penis extends to erectile dysfunction (ED) to conditions including sexually transmitted diseases (STDs) and cancer. To date, there has been little research evaluating vascular drainage from the penis. We aimed to evaluate penile blood flow in vivo and analyze its possible relationship with the lymphatic maker.

Materials and Methods: We established an in vivo system designed to assess the dynamic blood outflow from the corpus cavernosum (CC) by dye injection. To analyze lymphatic characteristics in the CC, the expression of Lyve-1, the key lymphatic endothelium marker, was examined by the in vitro system and lipopolysaccharide (LPS) injection to mimic the inflammatory conditions.

Results: A novel cavernography methods enable high-resolution morphological and functional blood drainage analysis. The expression of Lyve-1 was detected along the sinusoids. Furthermore, its prominent expression was also observed after penile LPS injection and in the erectile condition.

Conclusions: The current in vivo system will potentially contribute to the assessment of penile pathology from a novel viewpoint. In addition, current analyses revealed inducible Lyve-1 expression for LPS injection and the erection state, which requires further analyses on penile lymphatic system.

KEYWORDS
corpus cavernosum, erectile dysfunction, lymphatic vessels, Lyve-1, sinusoid

1 | INTRODUCTION

The penis is composed of the corpus cavernosum (CC), the urethra with fascial layers, nerves, blood vessels and lymphatic vessels.[1] The erectile tissue forms the CC and sinusoids, which are composed by endothelial cells, fibrous tissues, smooth muscles.[1–3] In response to sexual stimulation, the release of nitric oxide (NO) results in the formation of cGMP relaxing the smooth muscles. Penile erection increased blood inflow from the artery and decreased outflow from veins, which expands the sinusoids with blood.[4–6] Conversely, an enzyme termed PDE5 releases the blood from the CC to its normal, flaccid state. PDE5 inhibitors for treating erectile dysfunction (ED),

help to induce an erection.[7–11] Thus, the functions of the blood vessels involved in erectile function have been analyzed, and treatments for ED have advanced dramatically as part of vascular medicine. However, a significant number of patients with ED do not respond to these drugs mostly with unidentified reasons. While functional analyses of these areas have focused on erectile physiology, there has been little research on the mechanism of erectile maintenance and switching mechanisms from erection to the flaccid state. To evaluate further the mechanisms controlling the erection, a novel experimental system has been established in the current study. The system allows to visualize the process of penile blood drainage from the erection to the flaccid state.

We have previously reported on the histological features of penile CC and 3D reconstruction of histological images covering sinusoidal structures.[8,10,11] In general, cellular and histological changes of the penis have been suggested to correlate with ED. The blood vessels are reported as frequently impaired in such diseases. In addition, we have further examined several histological changes and makers related with the drainage or switch to the flaccid state. Hence, we analyzed lymphatic vessel maker to get clues to understand the potential causes of abnormalities in drainage system and refractory ED. Lymphatic vessels form an extensive network in the body and are important peripheral tissue organs to collect and drain excess lymph from extracellular sources to maintain tissue pressure and homeostasis.[12,13] The penile lymphatic system may also play significant roles in sexually transmitted diseases (STDs) and lymph node metastasis of penile cancer.[14–20] However, questions remain regarding the histological distribution of lymphatic vessels as well as their mechanisms for penile functions. In the current study, we evaluated the expression and status of Lyve-1, the key lymphatic endothelial maker, in the CC. Moreover, the penis is exposed to various micro-organisms from the external environment that may cause diseases such as STDs, which are known to deteriorate erectile functions.[21] However, the function of lymphatic vessels for such penile pathogenesis remains unclear. In the current study, we also utilized lipopolysaccharide (LPS)-treated mice to evaluate the time-course changes of Lyve-1 in the pathogenetic penis. In addition, we analyzed the dynamic expression status of Lyve-1 in the process of erection by inducing the CC to erection/flaccid state utilizing our culture system. Altogether, the current work discusses a novel in vivo system that can evaluate the penile blood outflow and novel evaluation of Lyve-1 expression status.

2 | MATERIALS AND METHODS

2.1 | Animals

ICR and C57BL/6J mice were purchased from CLEA Japan. They were maintained at the Animal Facility of Wakayama Medical University. Priapism phenotype model mice were utilized in the current study.[22] All mice were housed under controlled temperature (21°C) with a 12:12 h light–dark cycle. All procedures and protocols were approved by the Committee on Animal Research at Wakayama Medical University. We confirm that all the methods were carried out in accordance with relevant guidelines and regulations. The mice were anesthetized with three types of mixed general anesthesia (medetomidine, midazolam, and butorphanol) intraperitoneally.[23,24]

2.2 | Histology and immunohistochemistry

Penile tissues were harvested and fixed overnight at 4°C in 4% (w/v) Paraformaldehyde (PFA) dissolved in phosphate-buffered saline (PBS). The detailed protocols were previously described.[8,25–29] Serial sections (6 μm thickness) embedded in paraffin were prepared for immunohistochemistry.

The penile injection was previously described.[7] Mice were administrated with lipopolysaccharide (LPS; 129–05961, Fujifilm) in saline at the dose of 25 mg/kg.[30] After the general anesthesia, the LPS solution through the corpus cavernosum glandis (CCG) using a 29-gauge needle was slowly administered left intracavernously.

The procedures used to prepare the CC explants have been partly reported previously[7,9] The CC region was isolated by microdissections, removing the prepuce and dorsal vein, artery and nerve. Subsequently, urethra and corpus cavernosum urethrae (CCU) were removed to generate the CC explant. The isolated explant was less than 1 mm thickness with a smooth cutting cross-sectional surface. The harvested CC was cultured in Hank's balanced salt solution (HBSS). The CC was induced to erect/flaccid state by applying relaxation-inducing/contraction-inducing drugs (NO donor, phenylephrine).[7,9]

For immunohistochemistry after deparaffinization and rehydration, the slides were subjected to antigen retrieval using citrate buffer (0.1 mM, pH 6.0) at 121°C for 1 min. The following antibodies were used: anti-LYVE1 antibody (1:200, ab33682, Abcam), anti-FITC antibody (1:100, ab19224, Abcam), and anti-CD34 (1/200, RAM34, eBioscience™).

Immunostaining was visualized by fluorescent staining (1/200, Alexa Fluor 488-conjugated IgG and an Alexa Fluor 546-conjugated IgG, Thermo Fisher Scientific) and counterstained with Hoechst 33342 (1/1000, Sigma-Aldrich). A Vector® TrueVIEW® Autofluorescence Quenching Kit (SP-8400, Vector Laboratories) was used to eliminate autofluorescence. For visualization of anti-LYVE-1 antibodies, immunocomplexes were also detected with diaminobenzidine (DAB) staining. The tissue sections were counterstained with hematoxylin for 5 min. Images were acquired using a standard fluorescence microscope (BX51, Olympus®).

2.3 | Establishment of a novel mouse model of cavernography

After the general anesthesia, the root of the penis was occluded with a polyurethane tube (1 mm diameter) to interrupt blood flow. FITC-dextran (FITC-D, 2000 kDa, 60842–46-8, Sigma-Aldrich) with a

volume of 0.15–0.25 mL was injected through the left crus of the CC with a 29G needle until the penis was expanded (Figure 1A,B). We measured the width of the penis with digital calipers before and after injection. The visual analysis was adjusted to the midpoint of the CC from the tip of the CC to its root. After 15 min of continued expansion of the penis, the occlusion was released and the fluorescence intensity (FI) of the right CC was observed (Figure 1A,C). Unlike the human CC, the mouse CC is connected in the middle region.[8] When FITC-D was injected from the left side, the majority of the FI was observed to flow through the central part to the right side. Since the left side is subject to experimental manipulations and injection, we performed and collected visual image data using the right side with relatively less unaffected manipulation. We obtained time-lapse images every 10 s and analyzed the dynamic change of FI to monitor CC responses under a stereomicroscope. From the sequential images, the right side of CC was marked and the alternation rate of FI after the release of the occlusion was calculated using ImageJ software (Video S1).

2.4 | Quantitative real time-PCR (qRT-PCR) analysis

The harvested CC was cultured in Hank's balanced salt solution (HBSS). For achieving erectile condition, relaxation-inducing drugs (NO donor, acetylcholine, prostaglandin E1) were applied. For analyzing flaccid condition of the CC, contraction-inducing drugs (phenylephrine, endothelin 1) were used to induce vasoconstriction.[7,9] The CC just before introducing the contraction-inducing drugs was considered under erection conditions, and the CC 15 min after adding the contraction-inducing drugs was considered under flaccid conditions.[7,9] Total RNA was extracted from CC explants utilizing ISOGEN (311–02501, Nippon Gene). Complementary DNA (cDNA) was generated from extracted total RNA by PrimeScript RT master mix (RR036A Takara Bio). Quantitative real-time PCR (qPCR) was performed in the StepOnePlusTM Real-Time PCR System (Applied Biosystems) using the SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (RR820A, Takara Bio). Expression of lymphatic endothelial marker gene (*Lyve-1*) was normalized by glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).[31–33] The primer sequences are described below:

Lyve-1 forward, 5′-CACTAGGCACCCAGTCCAAG-3′.
Lyve-1 reverse, 5′-GTTGCGGGTGTTTGAGTGTC-3′.
Gapdh forward, 5′-AACGACCCCTTCATTGACCTC-3′.
Gapdh reverse, 5′-CCTTGACTGTGCCGTTGAATT-3′.

2.5 | Statistical analysis

For the analysis by measuring the width of the penis and qRT-PCR, either the Student *t*-test, followed by the *F* test, was performed. (values of $p < 0.05$ were considered to be significant).

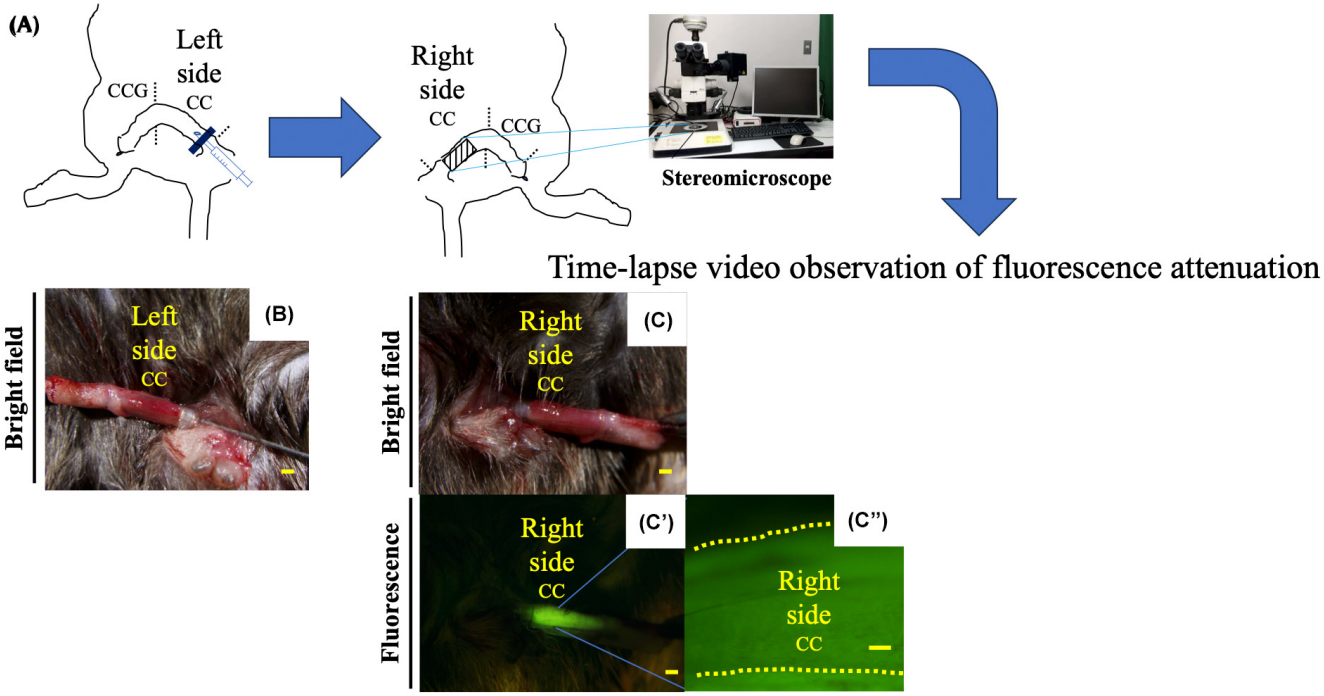

FIGURE 1 A novel in vivo cavernography system utilizing FITC-dextran. (A) An illustration showing the experimental diagram. Procedures and subsequent time-lapse analysis of fluorencet status are shown. (B) After general anesthesia, the mouse was placed in the supine position and the root of the penis was occluded by the polyurethane tube. FITC-dextran was injected through a 29G needle into the left CC under the tube. (C, C′, C″) After releasing the obstruction, we performed time-lapse images of the fluorescence signal changes in the central region of the right corpus cavernosum under a stereomicroscope. Corpus cavernosum (CC), corpus cavernosum glandis (CCG). Scale bar (B, C, C′) 1000 μm, (C″) 200 μm.

3 | RESULTS

3.1 | A novel cavernography to examine dynamic erectile responses in vivo

The mechanism of erection is complex and regulated through various circulatory factors in the penis. Such mechanisms have been clarified by electrophysiological experiments, primarily focusing on intracavernous pressure (ICP) measurement.[7–9,22] However, analyzing the dynamics of the penis has not been well performed. In previous research, we established an in vitro experimental system to analyze the dynamic contraction/relaxation responses of the penis.[7,9] In the current system, we aimed to analyze the dynamic changes of blood flow in the penis in vivo (Figure 1A). To visualize the penile blood flow in vivo, fluorescein isothiocianate-conjugated dextran (FITC-D) was utilized as a tracer. A novel cavernosography system in vivo was designed to assess the dynamic blood outflow from the corpus cavernosum (CC) after its injection into the penis, subsequently capturing the alternation of the fluorescence intensity (FI). After a tourniquet was applied to the root of the penis for occluding of the CC, FITC-D was injected via the left crus penis (Figure 1B). As the root of the penis was occluded, the FI remained in the CC after the injection. FITC-D injection significantly increased the width of the penis (2.52 ± 0.06[SD] mm) compared to the width before the injection (2.08 ± 0.03[SD] mm; Figure 2A). Cross-sectional views of the right side of CC, the site for observation, showed that the fluorescence signal was enhanced in the sinusoids, confirming the accumulation of FITC-D in the sinusoidal lumen (Figure 2B). After releasing the occlusion of the penis, right side of CC was observed under a stereomicroscope and the FI alternation was directly monitored and quantified in vivo (Figure 1C,C′,C″). FI almost completely disappeared 10 min later (Figure 2C). Concomitant with the disappearance of the FI, the width of the penis returned to the original width as before the injection. These results suggested that the novel FITC-D cavernography methods enable high-resolution morphological and functional analysis based on blood drainage with the fluorescent dextran dye. Since we have previously also established a priapism mouse model, we performed blood flow analysis utilizing the novel system to assess its applicability for pathophysiological understanding. Priapism is the continuous, unintentional erection that occurs in the absence of sexual stimulation.[22] The condition is significant because its severe forms of symptom result in extremely frequent ED.[34,35] After 10 min of obstruction release, priapism model mice still showed high FI in the CC (Figure 2C), which suggests a defective blood outflow as a pathogenic factor in the mouse confirming the efficacy of the current system.

3.2 | The histological distribution of Lyve-1 in the corpus cavernosum

We performed histological analyses of CC with sinusoids, which are critical structures for filling with bloods during the erection. The urethra is generally located in the ventral (lower) region of the entire external genitalia. A significant number of sinusoids were observed in the outer CC close to the tunica albuginea.[7–9,36] Several cell types localized to the CC were demonstrated as CD34, FLK1 (Fetal liver kinase 1 or VEGFR-2) positive endothelial cells located adjacent to the outer regions of the CC.[8,22] VEGFR-2 is mainly expressed in vascular endothelial cells, lymphatic endothelial cells.[31,37,38] In order to analyze the relationship between blood vessels and lymphatic vessel characters in the sinusoids, we analyzed the expression of Lyve-1 in the CC. Lyve-1 is a key marker that is predominantly expressed in the lymphatic endothelium.[32,33] The expression of Lyve-1 was mainly located along sinusoids of the outer CC adjacent to the tunica albuginea, where the expression of CD34 was located (Figure 3A,B). In addition, Lyve-1 expression is also prominent in sinusoids of the CC adjacent to the dorsal (upper) region of the urethra (Figure 3A,C).

3.3 | Lipopolysaccharide (LPS) injection induced the augmented expression of Lyve-1

The urethra serves as the gateway for the external entry of foreign materials. To observe the expression changes in the penis under inflammatory-mimicking condition, we injected LPS into the penis and observed changes in Lyve-1 in the CC. On the first day after administration, there was an increase of Lyve-1 expression, especially in the CC adjacent to the dorsal part of urethra (Figure 4B). By day 7 after the treatment, the observed changes returned to original levels similar to those before administration (Figure 4A,C). Tunica albuginea is known as necessary for drainage, which is comprehensible for the current Lyve-1 expression. The prominent Lyve-1 expression in the tunica albuginea region might indicate the character for drainage type of vessel. These findings suggest that Lyve-1 positive sinusoids may also play critical roles during inflammatory conditions (Figure 4D).

3.4 | The prominent expression of Lyve-1 in the erection state

We have previously established a novel in vitro CC culture system.[7,9] We have reported that the CC can be induced to the erection and flaccid condition by applying contraction/relaxation drugs in the culture system.[7,9] To get insights on the potential lymphatic character during the erection process, we analyzed the expression status by immunofluorescent staining during such process of erection and flaccid conditions. The marked expression of Lyve-1 was located in the erectile state compared to those in the flaccid state, especially in sinusoids of the outer CC adjacent to the tunica albuginea (Figure 5A,B). To further analyze these dynamic changes in Lyve-1 expression, we extracted RNA from both erection and flaccid states and performed real-time PCR. During erection, there was marked upregulation of the *Lyve-1* compared to the flaccid state (Figure 5C). These results suggest that the dynamic Lyve-1 expression and the

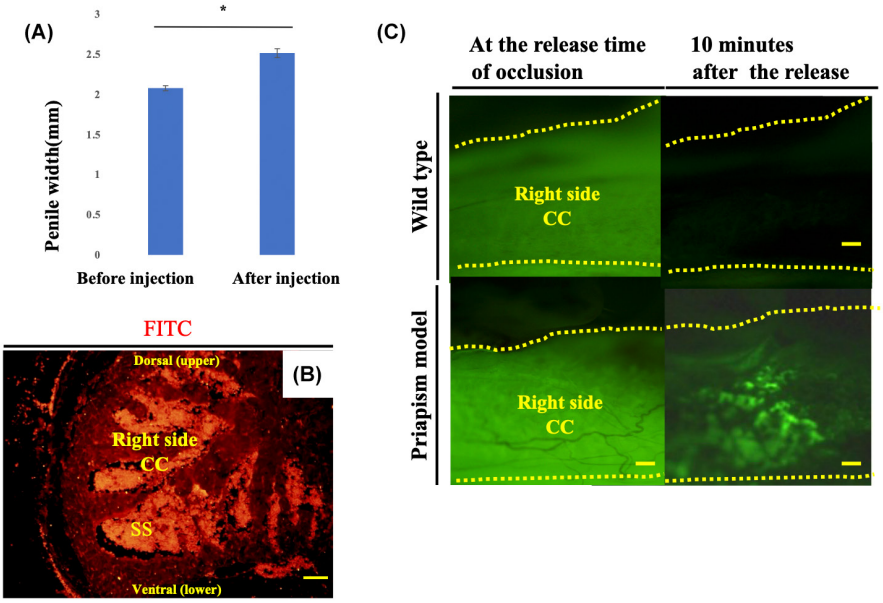

FIGURE 2 Demonstration of dynamic FITC-dextran signal alternation in the CC. (A) The width of the penis with digital calipers was measured before and after injection. After injection, the width of the penis (2.52 ± 0.06[SD] mm) was increased compared to that before injection (2.08 ± 0.03[SD] mm). (B) The signal of FITC in the right CC after injection of FITC-dextran into the left CC. The FITC-dextran injection filled the sinusoidal spaces. (C) The occlusion was released and the fluorescence intensity (FI) of the right CC was observed. Time-lapse images were obtained every 10 s and the dynamic change of FI was analyzed under a stereomicroscope. From the sequential images, the right side of CC was marked and the alternation of FI was calculated. After 10 min, the FI disappeared in wild-type mice. On the other hand, the FI remained in the priapism model mice. A time-lapse video analysis was performed by ImageJ software. CC, corpus cavernosum, SS, sinusoidal space, scale bar (B)100 μm, (C) 200 μm.

associated status may also be involved in maintaining the penile homeostasis during the erectile state (Figure 5D).

4 | DISCUSSION

4.1 | A novel in vivo visualization analysis for the blood outflow from the CC

An erection occurs when the smooth muscles of the penile corpus cavernosum (CC) are relaxed, penile blood flow increases concomitant with sinusoidal dilation and venous plexus compression.[39] Following sexual arousal, nitric oxide (NO) is distributed throughout the penis and causes relaxation of the smooth muscle and the penile erection.[7,9,40] In recent years, the increasing number of patients with sexual dysfunction have brought more attention on the mechanisms of erection towards the treatments of erectile dysfunction (ED).[41,42] ED is defined as the inability to have satisfactory sexual intercourse due to the inability to achieve or maintain an erection. It is estimated that at least 150 million men worldwide suffer from ED.[43,44] Although the association between diabetes mellitus and ED has been known for a long time, the relationship of lifestyle-related diseases such as hypertension, hyperlipidemia and obesity to ED has received particular attention in recent years.[45–48] The treatment of ED improved significantly owing to PDE5 inhibitors, but a significant number of patients showed resistance to the treatment.[42,49–51] Therefore, it is essential to analyze the causes of such severe forms of ED cases and to evaluate penile blood vessels/flow. The current clinical methods are mainly based on neurophysiological approaches and are not directly related to assess organic erectile functions.[52–54] The animal studies have elucidated the mechanisms of the erection mainly by the physiological experiments and have contributed to the development of some therapeutic methods.[55,56] However, the majority of such animal studies analyzed the changes in intracavernous pressure (ICP) measurement, which has been mainly assessed electrophysiologically.[22,57–61] Thus, there are no experimental systems that directly visualize penile blood flow even in such animal studies. Furthermore, the majority of such experiments are often performed on larger animals, and mechanistic analysis with genetically engineered model mice is largely underexplored. Therefore, a comprehensive understanding of the mechanism of blood homeostasis and pathophysiology has not been achieved.

Previous studies on erections have not investigated the relationship between the maintenance of erection and the flaccid state. We established an in vitro system to analyze contraction/relaxation in the CC.[7,9] In the system, isolated CC is micro-dissected to create tissue explants, followed by time-lapse imaging. By applying external factors such as phenylephrine and NO donors, inducible contraction/relaxation responses are analyzed, which revealed regional differences in the dynamic alternation of the sinusoids. We further focused on the vascular system of the sinusoids and now established an experimental system for in vivo blood outflow analysis. To assess

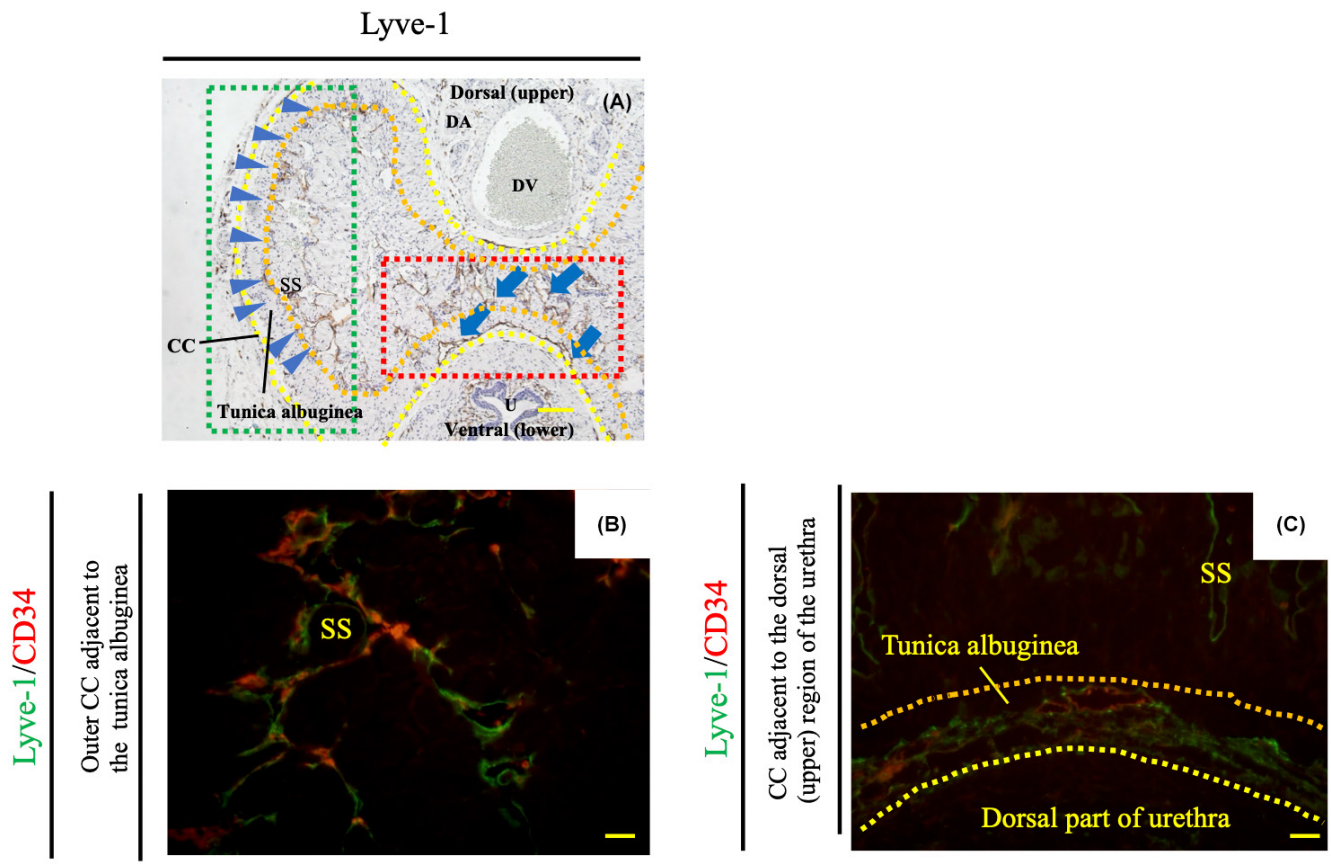

FIGURE 3 The characteristic mode of expression of Lyve-1 in the CC. (A) The expression of Lyve-1 in the DAB-stained CC sections. The yellow dotted line indicates the circumference of the CC and the area between the yellow and orange dotted lines represents the tunica albuginea. Green dotted line indicates the outer region of the CC. Lyve-1 was expressed along sinusoids of the outer CC adjacent to the tunica albuginea. (Blue arrowheads). The urethra is generally located in the ventral (lower) region of the entire external genitalia. Red dotted line indicates the CC around the dorsal (upper) region of the urethra. The expression of Lyve-1 was also located in the sinusoids of the CC adjacent to the dorsal part of urethra (blue arrows). (B) The expression of Lyve-1 was colocalized with CD34-positive cells in sinusoids of the outer CC adjacent to the tunica albuginea. (C) The expression of Lyve-1 was located in the sinusoids of the CC adjacent to the dorsal part of urethra. The colocalized expression of Lyve-1 and CD34 in the sinusoids surrounding the dorsal part of urethra was less prominent compared to that in the sinusoids of the outer CC adjacent to the tunica albuginea. CC, corpus cavernosum, sinusoidal space (SS), dorsal vein (DV), dorsal artery (DA), Scale bar (A) 100 μm, (B, C) 20 μm.

dynamic changes in blood flow during the erection process, we utilized FITC-dextran (FITC-D) for visualization. Its injection was designed to induce an erection with a technique frequently performed in the surgical treatment of Peyronie's disease, which displays abnormal penile bending. In addition to such induction of erection, FITC-D as s a tracer allowed real-time analysis of blood outflow dynamics from the penis. Thus, the current procedure has enabled the visualization of blood outflow from the sinusoids for the first time in vivo.

Regarding the usage of model mice, we have previously established a priapism mouse model and have identified one of the causative genes.[22] Priapism is a pathological condition with continuous abnormal erections, leading to ED if not treated promptly.[42,62–66] It has been speculated that priapism is caused by an imbalance between the inflow and drainage of blood flow from the CC. However, such an imbalance has not been experimentally assessed due to the inability of previous experimental systems. The current in vivo model revealed for the first time the defective drainage from the CC. By detecting the remnant blood flow abnormalities with priapism, we now provided a unique understanding of the pathophysiology using a different approach compared to previous experimental systems. Applying the current system to other penile disorders would enable real-time detection of abnormalities in the CC.

4.2 | The prominent expression of Lyve-1 was located along the sinusoids

The male external genitalia is frequently exposed to the outside environment and is often affected by poor hygiene. The external urethral meatus is a pathway for microbial invasion and exposure to pathogenic microorganisms can lead to urinary tract infections and sexually transmitted diseases (STDs). Risk factors for penile malignancies include foreskin and high-risk human papillomavirus (HPV) infection.[14–16] Penile cancer is also an important public health issue and its prevalence varies widely by region and race/ethnicity highly frequent in some countries.[67–69] Regarding such

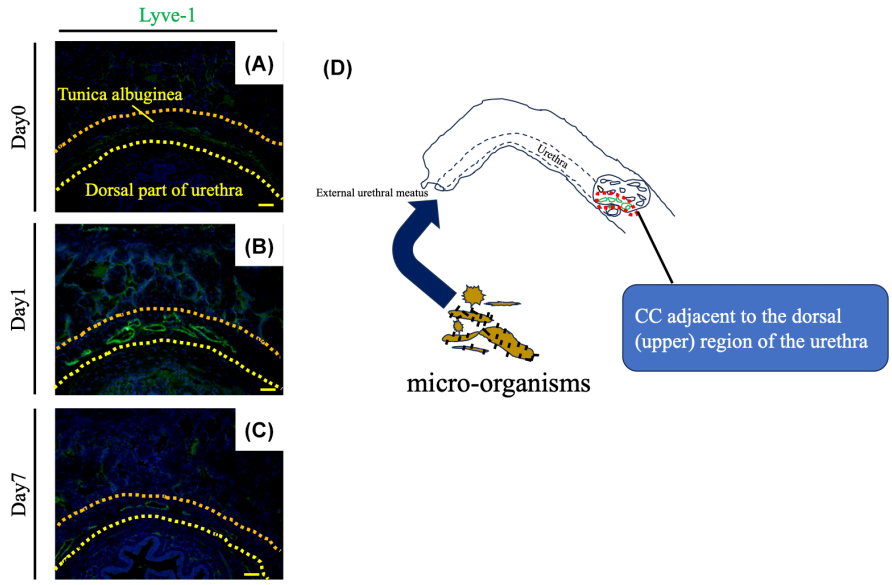

FIGURE 4 The expression of Lyve-1 after the lipopolysaccharide (LPS) injection. Mice were administrated with LPS in saline at the dose of 25 mg/kg. After the general anesthesia, the LPS solution through the corpus cavernosum glandis (CCG) using a 29-gauge needle was slowly administered left intracavernously. (A–C) The expression of Lyve-1, located in the CC adjacent to the dorsal part of urethra, showed an increase at 1 day post injection. By 7 days post-injection, the expression returned to the original level of the pre-injection. The yellow dotted line indicates the circumference of the CC and the area between the yellow and orange dotted lines represents the tunica albuginea. (D) An Illustration showing the possible link of the external urethral, external micro-organisms and CC. CC, corpus cavernosum. Scale bar 50 μm.

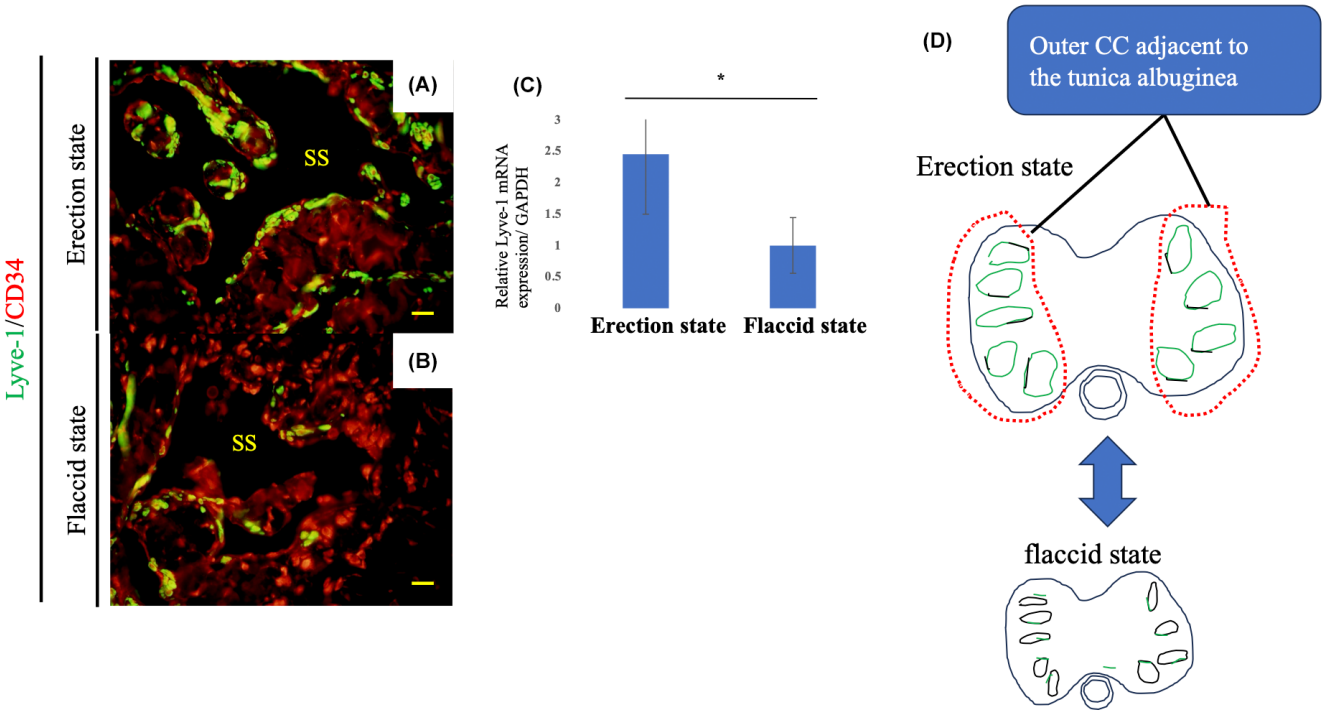

FIGURE 5 The expression of Lyve-1 in erection/flaccid state. We induced the CC into erection/flaccid states utilizing the in vitro CC culture system that was previously established.[7,9] (A, B) The expression of Lyve-1 was located in the outer CC adjacent to the tunica albuginea and was upregulated in erection compared to the flaccid state. (C) After inducing erection/flaccid state utilizing in vitro CC culture system, mRNA was extracted from CC explants. The augmented expression of Lyve-1 in the erection state compared to the flaccid state. (D) An Illustration suggesting the possible relationship between the CC and Lyve-1. SS, sinusoidal space. Scale bar 20 μm.

pathogenesis, lymphatic vessels not only play important physiological roles in homeostasis and immune response but also are involved in conditions such as inflammation and metastasis of malignant tumors.[17–20] Lymphatic vessels possess important pathway for lymph node metastasis of cancer cells. The prognosis of penile cancer is highly dependent on the status of lymph node metastasis, extending from the penis through the inguinal lymph nodes to the pelvis.[70–73] The control of inflammation via the penile lymphatic system caused by cancer and infection is important for maintaining homeostasis and immune responses. However, the histologic status of the penile lymphatic system remains unknown. With the novel cavernography system, we suggested the possibility that abnormal blood flow balance may contribute to some penile abnormalities.

Considering the possible involvement of lymphatic vessels in the regulation of blood flow balance, we also analyzed the expression of Lyve-1, the key lymphatic endothelial maker, in the CC. Recent discoveries of lymphatic endothelial markers such as Prox-1 and Lyve-1 have revealed a possible pathological regulation of lymphangiogenesis in various conditions involving tumor lymph node metastasis and inflammation.[32,74,75] We have also established a tail chronic stress model to conduct analysis focused on Lyve-1 and examined both blood vessels and lymphatic vessels.[76] Previously, we have performed an analysis of FLK-1 (VEGFR-2) expression in the CC.[11] The result showed its expression in sinusoids of the outer CC adjacent to the tunica albuginea. The expression of VEGFR-2 is observed not only in the vascular endothelial cells but also in the lymphatic endothelial cells.[37,38] To analyze the potential role of lymphatic characters in the CC, we performed immunostaining for Lyve-1 in the CC. The expression of Lyve-1 was detected along the sinusoids mainly in sinusoids of the outer CC adjacent to the tunica albuginea. In such region, significant number of sinusoids are located with the marked expression of CD34 and FLK-1.[7–9,11] In recent years, a possible unique type of vessels with characteristics and functions of both blood and lymphatic vessels has been detected in tissues, including Schlemm's canal in the eye, which functions to drain aqueous humor.[77] A recent report analyzed the expression of Prox-1 and Lyve-1 in CC, suggesting the possible existence of a unique vascular characters of both blood and lymphatic vessels by adopting different approaches of single cell analysis, which are consistent with the current results.[78] The current results suggested that Lyve-1 positive sinusoids in the CC may possess some sort of lymphatic as well as vascular characteristics.

4.3 | The expression of Lyve-1 may be involved in defensive roles in the penis

The external genitalia is generally exposed to external microorganisms potentially through the urethra, making the penis susceptible to various infections. However, a possible defense mechanism may prevent infections, which is still unknown due to lack of analysis. In the current study, the expression of Lyve-1 was also located along the sinusoids, primarily in the CC adjacent to the dorsal (upper) region of the urethra. In addition, we induced the experimental inflammatory conditions for the CC utilizing LPS injection and subsequently analyzed the time course for the expression of Lyve-1. The expression of Lyve-1 showed a marked increase after LPS exposure in the CC around dorsal part of urethra and subsequently returned to the pre-injection conditions 7 days after LPS injection. Lyve-1 plays a crucial role in inflammation and tissue remodeling.[79,80] For example, the cornea serves as the front structure for the external stimulus and Lyve-1 positive lymphatic vessels are induced during corneal inflammation.[80] In the major organs responsible for removing waste from the blood such as the liver and lungs, LPS stimulation indeed increased the expression of Lyve-1, suggesting its involvement as a potential role in the uptake of waste products and foreign substances circulating in the blood and lymphatic fluid.[79] Collectively, these results suggest that Lyve-1 positive sinusoids might function as a sort of defensive elements inside the penis. In the current paper, the interpretation of whether this means the presence of authentic lymphatic vessels or sinusoidal endothelium inherently expressing this unique character requires further experimental analysis.

4.4 | Dynamic changes of the expression of Lyve-1 during erection

In some species such as Aves, it has been reported that the penis can be induced to erect with lymphatic fluid. In fact, such erectile tissues have been referred to as corpus para-cloacalis vascularis or lymphatic spongy body.[81–84] The mechanisms and significance of such lymphatic copulatory organs are still under discussions but it is intriguing to consider the importance of tissue (lymphatic) fluids for protruding copulatory devices. It should be noted that erectile organs in such species are filled with lymphatic fluids from para-cloacal lymph nodes in the perineal region. In that aspect, the relationship with the erectile and lymphatic system might be an important link among species ranging for lymphatic copulation to lymphatic metastasis of penile cancer in pathogenetic conditions.

We have previously established an in vitro system where the CC enabled to induce into an erection/flaccid state.[7,9] In the current study, we further analyzed the expression changes of Lyve-1 during erection/flaccid state. The expression of Lyve-1 in the erection state showed markedly increased compared to that in the flaccid state. Moreover, its augmented expression of *Lyve-1* was confirmed by real-time PCR analysis. The expression of Lyve-1 is particularly elevated in sinusoids of the outer CC adjacent to the tunica albuginea. These regions have been reported to show increased expression of NG2, a pericyte-specific marker, which are known to play critical roles in vascular homeostasis.[85–87] Such regions may be involved in the venous compression that are critical for maintaining penile erection. The primary physiological function of the lymphatic vasculature is to generally maintain tissue

fluid homeostasis by receiving fluid and macromolecules from blood capillaries into tissue interstitial spaces.[88,89] The increased expression of Lyve-1 during the erection state may suggest the potential involvement of the lymphatic system in maintaining the penile homeostasis. Lyve-1 positive sinusoids may be involved in maintaining the blood flow within the CC which results in sustaining the erection. In the previously published paper, we reported early changes in gene expression in our system mimicking the erection/flaccid condition.[7–9] Further experiments are required to understand how the alterations in Lyve-1 are related to genes associated with erection. Another unique experimental approach using single cell analysis has recently reported a sort of unique feature of sinusoidal vessels and Prox1 expression in the CC has been noted to be decreased in aged mice compared to young mice.[78] Further analyses on blood vessels and lymphatic vessel characters in the penis may contribute to a more profound insight into erectile function and its abnormalities potentially lead to the discovery of novel therapeutic approaches.

ACKNOWLEDGMENTS

This work was supported by the Japan Society for the Promotion of Science grants 21K6822 and Research Fellow of Japan Society for the Promotion of Science grants 23KJ1859. The authors thank Norio Nonomura, Patricia Brennan, Timothy R Birkhead, Kentaro Suzuki, Yosuke Mukoyama, Masatsugu Ema, James Jester, Sang Won Han and Arthur L Burnett for their encouragements. The authors also thank all laboratory colleagues and Yugi Rim for their assistance.

CONFLICT OF INTEREST STATEMENT

Kota Fujimoto, Daiki Hashimoto, Sang Woon Kim, Yong Seung Lee, Takuya Suzuki, Masanori Nakata, Shinji Kumegawa, Shinichi Asamura and Gen Yamada declare that they have no conflict of interest.

ETHICS STATEMENT

Human rights statements and informed consent: This work does not contain human subjects.

Animal studies and Approval by Ethics Committee: All institutional and national guidelines for the care and use of laboratory animals were followed. All procedures and protocols were approved by the committee on animal research at Wakayama Medical University, Wakayama, Japan (approval number: 993, 1166).

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Fujimoto K, Hashimoto D, Kim SW, Lee YS, Suzuki T, Nakata M, et al. Novel erectile analyses revealed augmentable penile Lyve-1, the lymphatic marker, expression. Reprod Med Biol. 2024;23:e12570. https://doi.org/10.1002/rmb2.12570