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RESEARCH ARTICLE

Primo Vessel Stressed by Lipopolysaccharide in Rabbits

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Abstract
For tracking the primo vascular system, we observed the primo vessels *in vivo in situ* using the lipopolysaccharide (LPS) response in the lymphatic vessels of a rabbit. Injection of LPS (200 μg/kg) into the lymph nodes resulted in greatly stained primo vessels, which were swollen in some cases. We were able to obtain comparative images through alcian blue and diaminobenzidine staining, which clearly showed different morphologies of the primo vessels. The mechanism causing the response of the primo vessels to the injected LPS is still unclear; however, these results might be a first attempt at giving an

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explanation of the function of the primo vascular system and identifying the changes in the structure and function of the primo vascular system in response to an external stimulus such as an injection of LPS.

1. Introduction

Development of science today has a close relationship with human health and the dream of living an extended life. The meridian system is the basis of biomedicines in Oriental medicine [1]. The meridian system is responsible for circulation of energy through the body. In traditional Chinese medicine, acupuncture points on the human body form a mesh, called the meridian system, that connects them to one another, and their main function is smooth circulation of body energy to maintain health [2]. In Korea in 1960, research on the meridian system was started for the first time in the world. Since then, many research papers focusing on the meridian system have been published [3—9].

Since Kim [3] first announced the discovery of the Bonghan duct and described the meridians of acupuncture, many studies related to the primo vascular system (PVS) have been carried out to isolate and identify the components of the PVS and to determine their microscopic structures [4—7]. Our primo team has identified some morphological properties of the PVS, primo vessels (PVs), and primo nodes (PNs) using various anatomical experiments. Recently, visualization techniques using trypan blue and alcian blue have been developed, which have helped in the study of the PVS in mice and rabbits [5,6]. However, in many morphological observations of the PVS, only the features at a specific time were observed, even though a specific external stress was ongoing [7—9].

In this research, through the observation of the PVs inside the lymph vessels (LVs) of a rabbit, stressed by using lipopolysaccharide (LPS) treatment, we investigated the morphological modifications of those structures caused by an external stress [10,11]. We also compared two different images of the PVs stressed by using LPS, one image showing PVs stained with alcian blue and the other showing PVs stained with diaminobenzidine (DAB).

2. Materials and methods

New Zealand white female rabbits, weighing 1.5—1.8 kg and 10 weeks of age, were purchased from the Dae Han Biolink Company (Chungju, Korea). Prior to the experiment, the rabbits were fasted for 1 day or 2 days. The experimental procedures were approved by the Animal Ethics Committee of Sangji University, Wonju, Korea (approval code: 2014-16) [12,13].

Prior to the experiment, we prepared the following anatomical tools: anesthetic, alcohol, saline, electronic scales, electronic microscope, microtweezers, laboratory scissors, laboratory forceps, microtubes, etc. [12,13]. At dissection, the following three points were carefully observed: (1) periodically, saline (40°C) was poured evenly over the organs to help in the circulation of fluids; (2) if bleeding occurred, gauze was applied and pressed to staunch the wounded area; and (3) when we separated the membrane with forceps, we proceeded carefully so that anatomical bleeding did not occur. To observe the PVs via the anatomical experiment, we carried out the following four steps.

2.1. Rabbit preparation

(i) The anesthetic, a mixture of zoletil (0.5 mL) and rompun (2.5 mL), was injected into the leg muscles of the rabbits using a 3-mL hypodermic syringe.
(ii) A hair trimmer was used to shave off all abdominal hair.
(iii) The rabbits were placed on the laboratory table, and its arms and legs were secured with Velcro belts.

2.2. Surgical procedure

(i) In order to disinfect the abdomen, we sprinkled about 1—2 mL of 70% ethanol over the top of the skin and wiped the skin with an aseptic tissue.
(ii) Using toothed forceps, we gripped the middle skin of the abdomen and used surgical scissors to incise the outermost skin along the middle line (linea alba line) of the abdomen down to the symphysis pubis and up to the episternum.
(iii) We made a 1-cm incision on the linea alba line in the straight muscle of the abdomen on the lower one-third of the line from the episternum to the symphysis pubis.
(iv) We moved the internal organs to the preferred side (right or left) to reveal the lymphatic vessels in the abdomen. Then, we covered the organs with wet gauze and continuously sprayed a warm saline solution over the top of the gauze to avoid dryness.
(v) If the bladder had much urine, we extracted the urine using a syringe.

2.3. Staining with alcian blue and DAB

(i) Prior to staining, we injected LPS into the lymphatic vessels of the rabbits so as to observe the PVs.
(ii) We selected an injection point along the LV, and in 30 seconds, we injected a small amount (0.03—0.1 mL) of preloaded 1% alcian-blue-staining dye into the LV using a 31-gauge ultrafine insulin syringe.
(iii) We waited for 5 minutes, and then we injected DAB solution, with or without alcian blue staining. After 5 minutes, we were able to observe the PVs inside the lymphatic vessels and the PNs linked to the PVs.
(iv) We divided the rates into five experimental groups, each group having a different combination of the three procedures: LPS injection, alcian blue staining,

Table 1 Five experimental groups for the different combinations of the three procedures (LPS injection, alcian blue staining, and DAB staining) and the number of rabbits in each group.

Combination groups	Procedure of injection & staining			No. of rabbits
	LPS	Alcian blue	DAB	
A	×	O	×	6
B	O	×	×	3
C	O	O	×	4
D	O	O	O	4
E	O	×	O	4
Total numbers of rabbits				21

DAB = diaminobenzidine; LPS = lipopolysaccharide; O = yes; × = no.

and DAB staining. The groups and the number of rabbits in each group are shown in Table 1.

2.4. Observation and extraction of PV

(i) We used microscopic observations to identify the stained PVs inside the LVs. PVs shaped like threads were found inside the washed LVs.
(ii) If the LVs were clearly visible, we carefully extracted them so that we could isolate the PVs using forceps. In general, the separated PVs were curled.

3. Results

Prior to extracting the PVs using their response to the LPS injection, the morphological and structural characteristics of the PVs, such as their thickness and lengths, connected to suspected PNs were obtained. Some representative photographs of typically long PVs for Combination Group A in Table 1 are shown in Fig. 1. Several PVs were observed in rabbits using an alcian blue solution, which flowed into the lymph node and slowly exited at an abdominal LV. The lengths and diameters of the LVs were of the orders of a few centimeters and a few hundred micrometers, respectively [13].

Fig. 1A shows branched PVs and Fig. 1B shows a long, thread-like PV. Prior to isolation from the LVs, several PVs were seen to branch from one PN. The extracted and isolated PVs had an average measured length of about 10 mm and an average diameter of about 25—35 μm. The staining dye existing in most of the LVs with lymphatic fluid dissolved over a period of time. Thus, the only clearly stained area that was observed was the PV. The PV's structure could be seen because the dye had attached itself to the wall of the PV and did not interfere with the flow of the lymph fluid. Owing to the flow of the dye in the LVs, the PV could be observed easily [14,15].

As shown in Figs. 2A—C, after the injection of LPS into the LVs, the PVs were seen clearly. Prior to injecting the LPS, observing the PVs in the LVs was not easy. Figs. 2A and B are relevant to the experimental results for Combination Groups B and C in Table 1, respectively. The LPS was injected into the lymphatic node, and it flowed into and through the LV, showing the PVs floating in the center of the LV, as shown in Fig. 2A. Fig. 2B shows one long, thread-like PV and a lymphatic valve inside an LV with alcian blue staining. The magnified views depicted in Fig. 2C clearly show one PV and several lymphatic valves connected to lymph nodes. Using LPS, we were able to separate the PV from the LV and to investigate the physiological and pathological features of the PV [16,17].

The PVS sample taken from the LV was placed on a slide after washing with phosphate buffer solution. Fig. 3A shows one optical image, under a white lamp, of the PV within an LV for Combination Group D in Table 1. Fig. 3B shows three fluorescent optical microscope images of an isolated alcian-blue-stained PV and LV sample (indicated by the rectangular box in Fig. 3A), obtained using blue, green, and red filters after DAB staining of the sample. The diluted fluorescent microscopic spots are distributed and confined inside the PV. As shown in Fig. 3B, the fluorescent image of the PV under the blue filter is brighter than that under the other two filters after DAB treatment of the alcian-blue-stained specimen.

Figs. 4A and 4B show two optical images of a PV under three filters (blue, red, and green) after DAB treatment of a

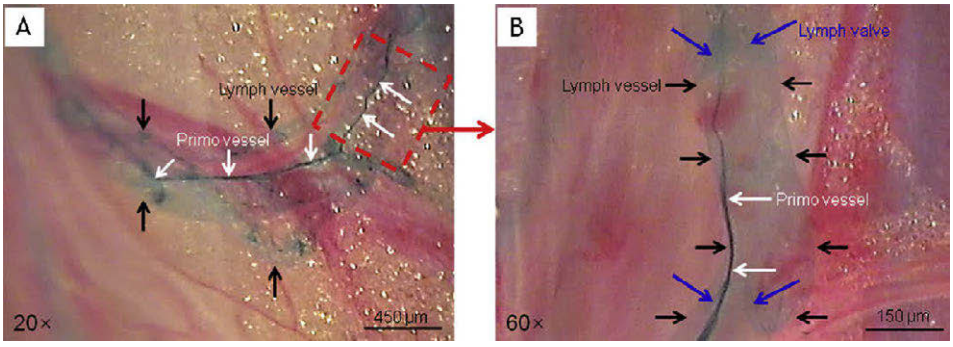

Figure 1 Typical PVs (white arrows) and LVs (black arrows) are active during the rabbit's respiration without LPS inflammation for Combination Group A. (A) The branched PVs inside the LVs, which were attached to organs, were stained using alcian blue. (B) A magnified view of the broken red rectangular box in Fig. 1A showing a thread-like PV inside an LV leaving the organ, and several lymphatic valves (blue arrows). The black arrows show the boundaries of the LVs. LPS = lipopolysaccharide; LV = lymph vessel; PV = primo vessel.

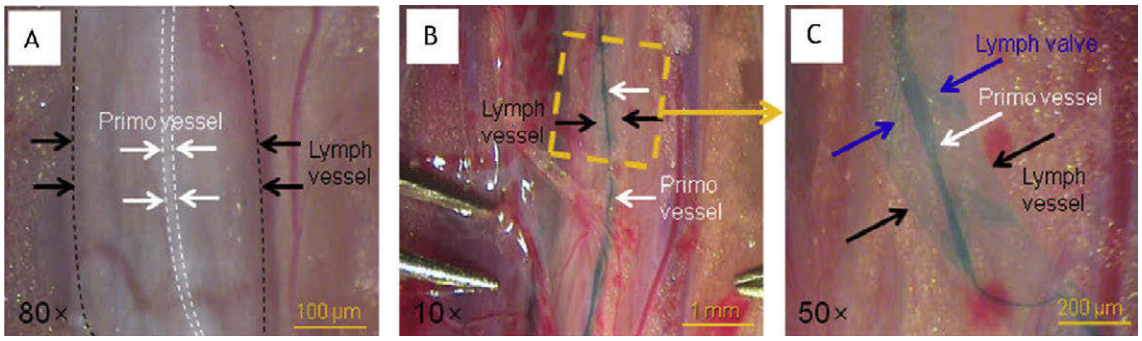

Figure 2 (A) Specimen without alcian blue staining shows that the PV (white lines and arrows) was clearly positioned in the center of the LV (black arrows) after injection of LPS into the LV for Combination Group B. (B) Specimen shows the PV stained with alcian blue dye after the injection of LPS for Combination Group C. (C) A magnified view of the rectangular box in Fig. 2B, showing a PV, an LV, and several lymphatic valves (blue arrows). LPS = lipopolysaccharide; LV = lymph vessel; PV = primo vessel.

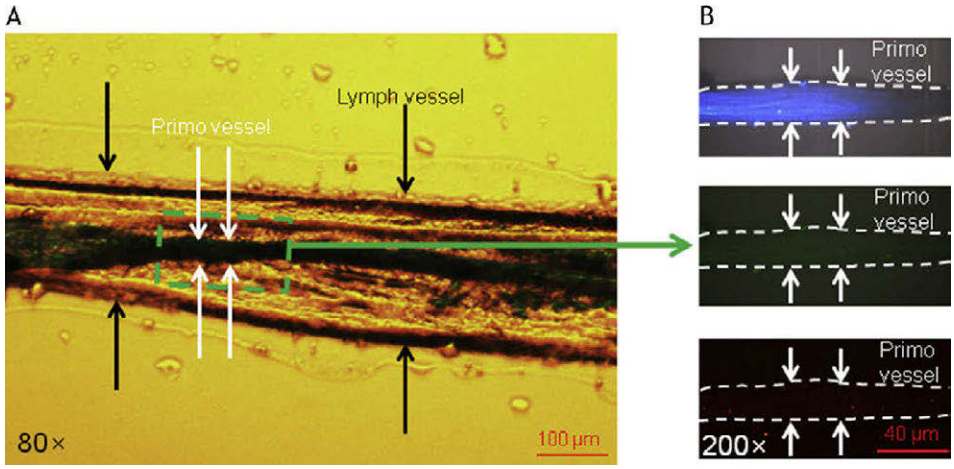

Figure 3 (A) Optical image, under a white lamp, of a PV within an LV after LPS treatment for Combination Group D. (B) Three optical images, obtained by using a blue, a red, or a green filter, after DAB treatment of the alcian-blue-stained specimen after isolation of the PV and the LV. The diluted fluorescent microscopic spots in the magnified views of the PV, inside the rectangular green box in Fig. 3A, are confined inside the PV. DAB = diaminobenzidine; LPS = lipopolysaccharide; LV = lymph vessel; PV = primo vessel.

specimen that had been injected with LPS to induce an inflammation response and then stained with alcian blue. These are relevant to the experimental results for Combination Group E in Table 1. The diluted fluorescent images were observed by using blue, red, and green filters, as shown Fig. 3B, and the probability of observing a clear fluorescent image for PVs inside LVs was increased by more than 90%. We obtained brighter fluorescent images of the

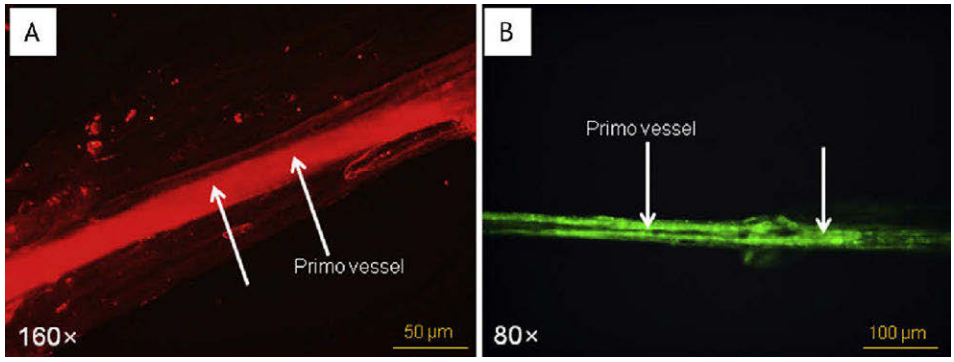

Figure 4 Two optical images of a PV (white arrows) for Combination Group E, obtained by using (A) a red and (B) a green filter. These specimens, which were obtained after DAB treatment and without alcian blue staining, show that LPS treatment was more effective in inducing an inflammation response. DAB = diaminobenzidine; LPS = lipopolysaccharide; PV = primo vessel.

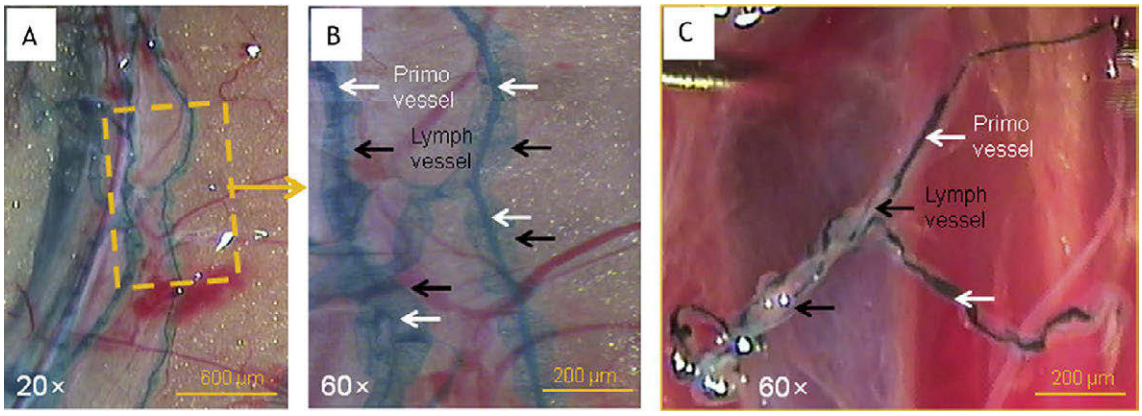

Figure 5 (A) PVs taken from the LVs after LPS treatment and alcian blue staining were placed on a caudal vena cava and observed using an optical microscope. (B) Magnified view of the PVs (white arrows) and LVs (black arrows) present inside the orange rectangular box in Fig. 5A. The PVs of the PVS appear to be thread-like bundles stained with alcian blue and floating inside the LVs. (C) Isolated and extracted PVs and LVs visible as strips and spiral lines, respectively. LPS = lipopolysaccharide; LV = lymph vessel; PV = primo vessel; PVS = primo vascular system.

PV under blue, red, and green filters. In order to track more PV connectomes for the body's meridian system, we suggest a new staining protocol, which uses LPS treatment to induce an inflammation response, for observing PVs after DAB treatment without alcian blue staining.

We studied the biological effects on PVs caused by an inflammation response induced by the injection of 200 μg/kg of LPS. The findings of this study and the experimental approaches used here may help explain the structure and function of the PVS in normal and diseased subjects in future studies. Fig. 5 shows an optical microscopic image of an abdominal LV in a rabbit's caudal vena cava region after treatment with LPS. The PVS sample taken from the LV was placed on a caudal vena cava and observed using an optical and fluorescent microscope, and the results are shown in Fig. 5A. Fig. 5B, which is a magnified optical microscopic image of the specimen shown in the orange rectangular box of Fig. 5A, shows thread-like bundles of PVs stained with alcian blue and floating inside an LV.

The isolated and extracted LVs and the PVs were nearly separated with two different colors, as shown in Fig. 5C, but were visible as strips and spiral lines, respectively. However, the PVs show various bundles, colored blue by the alcian dye. The PVS structures, white microvessels, measured about 25–35 μm in diameter. The average diameter of the LVs inside the caudal vena cava was about 150–200 μm, which was fairly uniform for all samples. The average length of the PVs from the LVs was about 5.0–10.0 mm, and this was fairly uniform throughout the samples [11,12].

4. Discussion

PVs were found by performing a microdissection experiment on rabbits, and the morphological characteristics of those PVs could be observed after the injection of LPS into a rabbit's lymphatic node. However, in some cases, the LPS injection did not work, which depended on the condition of the rabbit, so we made a filtered alcian-blue-staining solution. The probability of observing PVs after isolation and dissection was increased by more than 90%. After DAB treatment, the fluorescent image of the PV under a blue filter was brighter than those under the red and green filters, which was due to the blue color caused by the alcian blue dye. In order to track more PV connectomes for the body's meridian system, we suggest our new staining protocol, which uses LPS treatment to induce an inflammation response, for observing PVs after DAB treatment, but without alcian blue staining.

LPS treatment is necessary for reproductive isolation of PVs in rabbits. The goal was to track the transmission pathway, which might follow the route of the PVS, using LPS to stimulate the acupuncture meridians, as shown in the figures. PVs could be easily observed inside the LVs of rabbits after injection of LPS. Our findings may help in classifying the structures and functions of the PVS in normal and diseased patients in the future. For the study of the primo circulatory system, this method of finding a connectome of a PV in a living body is very meaningful and needs to be investigated further.

Disclosure statement

The authors declare that they have no conflicts of interest and no financial interests related to the material of this manuscript.

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