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CONTRAST-ENHANCING OPTICAL METHOD TO OBSERVE A BONGHAN DUCT FLOATING INSIDE A LYMPH VESSEL OF A RABBIT

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ABSTRACT

Novel threadlike structures, so-called Bonghan ducts (BHDs), were recently rediscovered inside large caliber lymphatic vessels using two different staining dyes in rabbits (Janus green B and Alcian blue) and fluorescent nanoparticles in rats. These three methods have the drawback of injecting chemical agents into the lymphatic vessels, which might damage the BHD and hinder further investigation of its physiological function. New methods to observe BHDs without using external chemical agents need to be developed. In the present work, we introduce a contrast enhancing optical method for in vivo observation of BHDs floating inside large caliber lymph vessels. The method uses a low-pass filter above about 650 nm, with an arrangement to minimize the light reflected from the surface of the lymph vessel. We captured films showing movement of a BHD as the animal respired. Applying the previous Alcian blue injection technique, we obtained BHD samples from the lymph vessel and observed the distribution of rod-shape nuclei (the essential feature of a BHD). BHDs can now be observed inside lymph vessels by using contrast-enhancing instrumentation without visualizing chemical agents.

Keywords: Bonghan corpuscle, Bonghan duct, lymph vessel, Optical method, Low-pass filter

Rapid progress in understanding the lymphatic systems has drawn much attention from a large general biomedical audience to puzzles about lymphatic development, growth, and functions under normal physiologic conditions and during disease processes. For example, lymphangiogenetic discoveries, such as lymphatic growth factor/ligands, endothelial receptors, transcription factors, and genes, were (and are) hot topics, and translation of these discoveries into the clinical arena have been a subject for reflection (1). Relationships between tumors and the lymphatic system are another area of serious study in this field (2). With all the recent progress in molecular understanding of the lymph system, it may seem quite inappropriate to now ask if we fully know the macroscopic anatomy of the lymph system. Anatomy has been taken for granted by those who rushed to microscopic-level research. It is, nonetheless, valuable in this molecular age to reflect upon the basic premises for anatomic structures upon which physiological functions are based. It is probably inconceivable that a threadlike structure floating in the lymphatic fluid, which has not been noticed in situ due to its transparency and has not been captured in conventional anatomical investigation dealing with dead bodies, actually exists. This structure was first observed by Bong Han Kim in the early 1960's, and he suggested that it was another lymphocyte-

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generating organ (besides the well-established bone marrow) (3). If such a novel structure indeed exists, it certainly would call for intensive investigation of its relation to the normal and pathological functions of the lymph system.

Bong Han Kim claimed that the threadlike structures existed throughout the body as a novel circulatory system, but his claim has not yet been confirmed. Only Fujiwara and some of us were able to follow his works on novel floating structures inside blood vessels and structures on internal organs (4-7). However, even Fujiwara failed to confirm the floating circulatory structures inside lymphatic vessels. As far as we know, we, for the first time, developed a new visualization method by using Janus green B, with which we were able to demonstrate the floating structure called a lymph Bonghan duct (BHD) and Bonghan corpuscles (BHCs) (8). Soon after our finding, we developed another method, using magnetic fluorescent nanoparticles, to detect BHDs in rat lymph-vessels (9). Recently, we also developed a more effective method using Alcian blue to demonstrate these structures inside rabbit lymph vessels (10).

However, some skepticism exists that the structures are just artifacts induced by the injected chemical agents. In addition, the methods using chemical dyes had the drawback of severely damaging the BHD and the BHC. The functional studies on BHDs and BHCs required intact specimens not damaged by chemicals and could not be performed. These drawbacks led us to develop an optical method, without injecting chemical agents into the lymph vessels, to directly observe the BHDs and BHCs floating inside the lymph vessels of rabbits.

In this article we report a contrast-enhancing optical method with which we were able to observe BHCs and BHDs in rabbits without any visualizing chemical agents. In addition, we show an image of a BHC and a BHD taken from a lymph vessel. The specimen was shown to have the hallmark characteristic of a BHD, namely, an aligned distribution of rod-shaped nuclei (5,7).

METHODS AND MATERIALS

Animal Preparation and Surgical Procedure

New Zealand White rabbits of 1.5 kg ~ 2.0 kg were obtained from Jung Ang Laboratory Animal Company (Seoul, Korea). The animals were housed in a temperature-controlled environment (23°C) with 60% relative humidity, applying a 12-hr light/dark cycle. The animals had ad libitum access to food and water, and the procedures involving the animals and their care were in full compliance with the institutional guidelines of Seoul National University and current international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996). The rabbits were anesthetized intraperitoneally with urethane (1.5 g/kg), and all surgical procedures were performed under general anesthesia. Under deep anesthesia, the abdominal side of rabbits was opened, and large vessels in the skin of the abdomen and the thorax were clamped to minimize blood flow over the organ surfaces. Lymphatic vessels around caudal vena cava and iliac veins were studied.

Contrast-Enhancing Technique to Reveal Bonghan Structures Inside Lymphatic Vessels

In order to visualize BHC and BHD inside lymphatic vessels of rabbits, we first covered a halogen lamp with red cellophane paper and the wavelength of transmitted light was measured with a spectrometer (HR4000CG-UV-NIR, Ocean Optics, UK). As shown in the inset of *Fig. 1*, the cellophane paper functioned as a long-wavelength pass filter around 600 nm. The light and stereoscope were positioned such that the light reflected from the lymphatic vessels and surrounding fat to the objective lens

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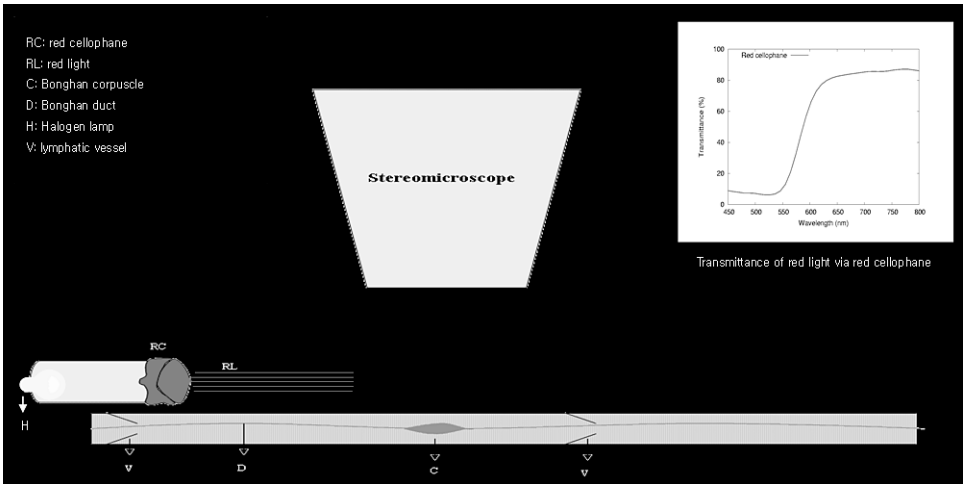

Fig. 1. Observational set-up with red light from a halogen light source over a lymphatic vessel of a rabbit in a dark room. The red light transmitted through red cellophane paper ranges over 600 nm, as shown in the inset. V: lymphatic valve, C: Lymph Bonghan corpuscle, D: Bonghan duct, RC: red cellophane paper, H: halogen lamp.

was minimal. Under stereomicroscopy, we exposed lymphatic vessels around abdominal arteries and veins, and removed fat from around the lymphatic vessels using micro-scissors and micro-forceps Observation was done using a stereomicroscope (Olympus SZX12, Japan) and recorded using a CCD camera in situ and in vivo (Olympus DP70, Japan) as illustrated in *Fig. 1.*

Sampling and Examination with Confocal Laser Scanning Microscopy

In order to verify that the optically-identified threadlike structure was real, we injected Alcian blue into the lymph vessel following the method described in our previous work (10). We removed the visualized BHC and BHD from the lymph vessel and stained with acridine orange for visualization of nuclei using a confocal laser scanning microscope as previously described (5). In addition, we also injected a fluorescent dye (DiI 1.1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate) into the lymphatic vessel to strongly stain BHD and BHC for in situ observation with a stereomicroscope (MVX10, Olympus, Japan).

RESULTS

We successfully illuminated lymph vessels with our new optical device and observed Bonghan ducts floating inside large lymph vessels near the caudal vena cava of rabbits (*Fig. 2*). The lymphatic BHDs were floating in the middle of the lymphatic flow and moved up and down as the rabbit respired under deep anesthesia. This image was obtained with our new optical method by improving the contrast without injecting any staining dye. Nevertheless, the shape, size, and color of the BHD were consistent with the previous data taken after injection of a visualizing agent (8-10). Using this contrast-enhanced optical method, we were able to detect otherwise hardly visible BHCs and BHDs, and their morphometric data are given in *Table 1.* We examined six rabbits and specimens of eight BHDs and six BHCs were obtained. The BHCs were of oval shape with a size of about 700 µm x 200 µm and were connected at both ends by BHDs whose diameters were about 30 µm.

The BHD and BHC could be drawn out from the lymphatic vessel with forceps (*Fig. 3*). For this purpose, Alcian blue was

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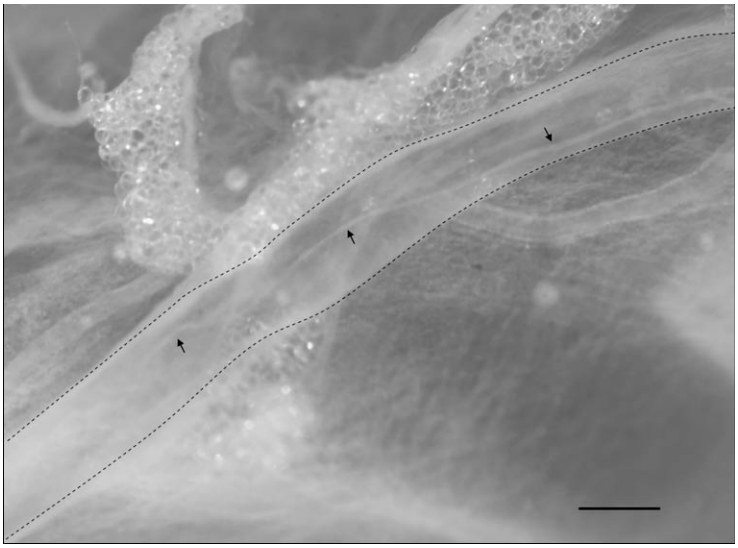

Fig. 2. Stereoscopic image of a lymphatic vessel on the caudal vena cava of a rabbit. This picture shows the lymph Bonghan duct (arrows) inside the lymphatic vessel (dotted line). The scale bar is 500 µm.

TABLE 1
Size Data on Bonghan Ducts and Bonghan Corpuscles Inside the Lymphatic Vessels of Rabbits

Subject number	Weight (kg)	W (µm)	N	Lx (µm x µm)	D (µm)	d (µm)
1	1.4	500	1	1500	44	44
2	1.4	544	0	n	30	30
3	1.5	662	1	485x265	30	30
4	1.0	350	1	690x170	35	35
5	1.8	570	1	390x109	18	17
				500x128	36	35
		714		500x100	26	21
6	2.0	500	2	1000x143	43	43
		314		n	29	29
Average	1.5	519	1.2	723x173	32	32
Std	0.3	138	0.8	397x78	8	9

The abbreviations are as follows: W, diameter of lymphatic vessel; D and d, diameters of the Bonghan duct's thickest and thinnest parts; N, number of observed corpuscles; L and l, long and short diameters of oval-shaped corpuscles; n, not measured.

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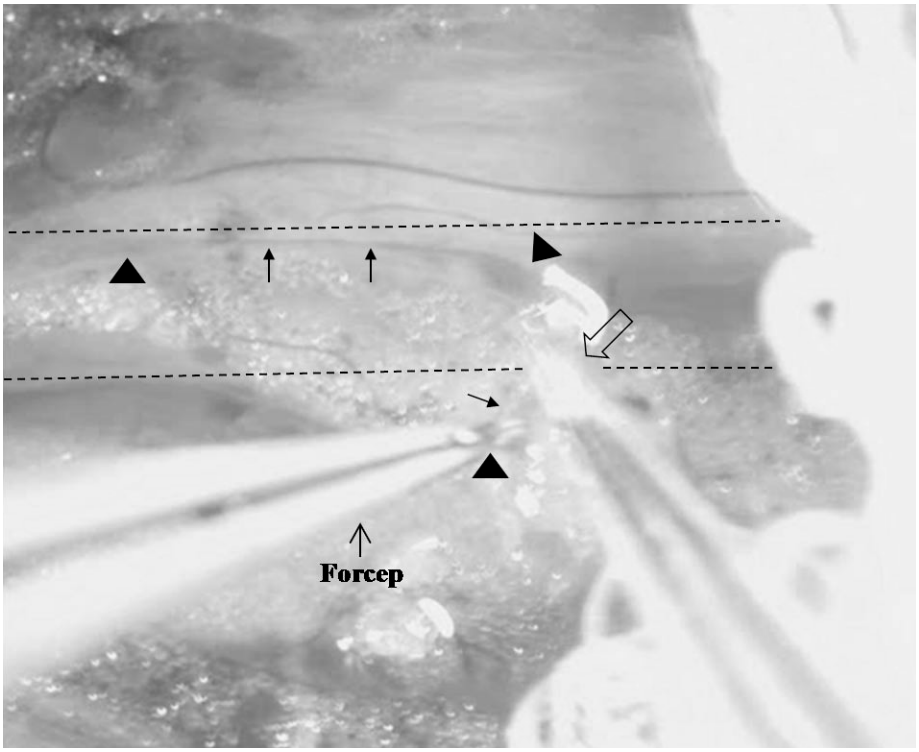

Fig. 3. Pulling out a BHD from inside a lymph vessel. A BHD (arrows) with BHCs (arrow heads) was taken by using forceps and was pulled out through on opening (open arrow) in the lymph vessel (dotted lines). The BHD and the BHCs were stained blue by injecting Alcian blue into the lymph vessel. The lymphatic vessel was so transparent that we needed to indicate the boundaries by using dotted lines.

injected into the lymph vessel to stain BHD and BHC. A small portion of the lymphatic vessel wall was incised, and forceps were inserted to hold and draw out the well-visualized BHC.

Specimens were further analyzed histologically to examine their morphologies. One of the hallmarks of a BHD is the presence of rod-shaped nuclei. The lymphatic BHD displayed these nuclei following DNA staining with acridine orange and confocal laser scanning microscopy images (*Fig. 4*).

Very rarely a BHD which exited the lymphatic vessel wall can be traced. In *Fig. 5*, a BHC and BHD stained with DiI demonstrate a BHD that comes out of the right-hand side of the BHC, meets the vessel wall, and continues to enter the nearby fat tissue.

DISCUSSION

The key technical point in our optical method is arranging the red light, as shown in *Fig. 1* to minimize reflected light from the lymphatic wall and surrounding fat tissues. We could not determine why this red light worked well for visualizing the floating BHD and BHC inside the lymph vessel. It may be that the long wavelength of red light (see spectrum in *Fig. 1*) penetrated deeply inside the lymphatic vessels. Once the floating structures in the lymph vessels had been detected, we were then able to recognize them even with the white light of a halogen lamp. However, in the beginning, noticing the presence of the floating structures without using a contrast-enhancing red beam was extremely difficult.

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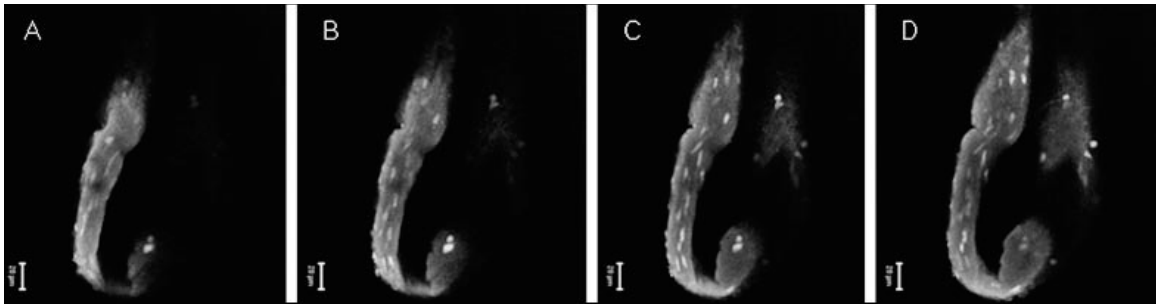

Fig. 4. Optical sections and rod-shaped nuclei. A BHD specimen was stained with acridine orange, a DNA staining dye, and an image was taken with a confocal laser scanning microscope. Panels A, B, C, and D are for a 10 µm-depth difference in sequel as optically sectioned. The aligned distribution of rod-shaped nuclei (green fluorescence color) is a hallmark of a BHD as seen in many other samples from various sources, such as blood vessels, organ surfaces, and brain ventricles.

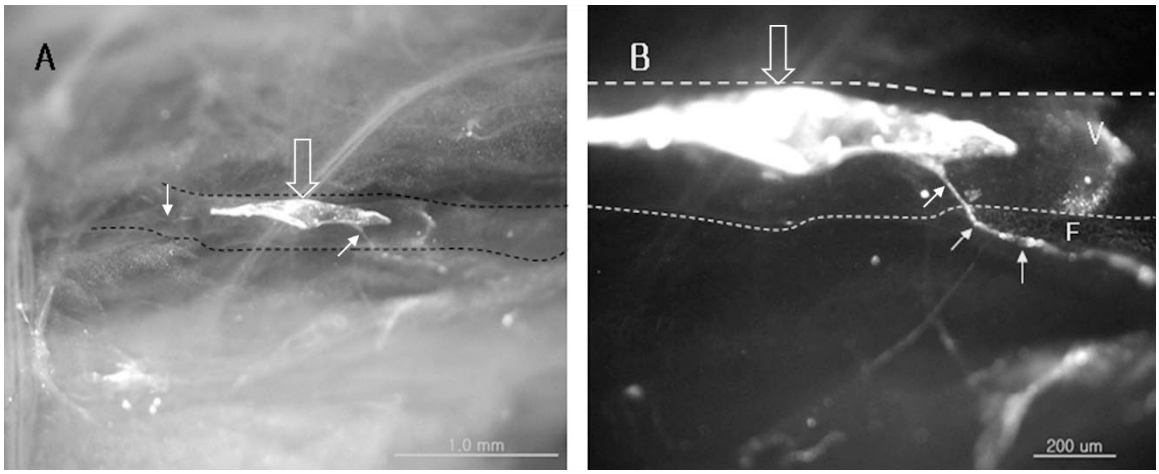

Fig. 5. Fluorescence image of a BHC (open arrow) and a BHD (arrows) stained by DiI, which was injected into a lymph vessel (dotted lines) around the caudal vena cava of a rabbit. The image is a merge of bright-field and fluorescent images. The injected DiI flowed away with the lymph fluid, but stained the BHC, and its associated BHD, strongly. The most notable thing was that the BHD came out through the lymph vessel wall and entered the surrounding fat tissue (F). V is a, valve that was weakly stained by DiI. Panel B is a magnified view of A.

A practical drawback in our new method is that most of the surrounding tissues, such as fat around the lymph vessels, need to be removed for visualization. Usually, it took about one hour to approach the lymphatic vessels by removing the surrounding fat and to visualize the BHC and BHD. We were able to take only one or two specimens from each rabbit because other lymph vessels had already become too degraded to be investigated properly.

The current technique is state-of-the-art for visualizing a lymphatic BHD. Manipulating a BHD to remove it from inside the lymph vessel is difficult with such low visibility. Thus, as shown in *Fig. 3*, we injected Alcian blue to color the BHD and BHC blue, and we obtained samples by drawing them out from the lymph vessel. If a sample is to be examined to ensure that it is a BHD, the minimal necessary condition is to document the distribution of rod-shaped nuclei (as shown in *Fig. 4*).

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Questions can be raised whether the observed BHD might be produced due to abnormal conditions such as the anaesthetic or surgical processes. If such a threadlike structure was produced in the lymphatic flow, it must be some coagulation of the contents of the lymphatics. One possibility is fibrin connecting lymphocytes in the lymph. This is unlikely as seen in *Fig. 4*. If the threadlike structures were fibrins with lymphocytes, the shapes of nuclei should be mostly oval or spherical instead of rod-shape because fibrins do not have nuclei, and lymphocytes have round nuclei. Furthermore, the alignment of rod-shaped nuclei as shown in *Fig. 4* is a characteristic feature of other BHDs which were found in other parts of animal body that have neither fibrins nor lymphocytes such as the surface of internal organs (7) or the spinal cord of rabbits (6).

Various questions on the presence of this new anatomic structure inside lymph vessels need to be answered in the future. First, the locations of the starting and the ending points of the threadlike BHDs have yet to be determined. According to Kim (3), BHDs form a network of a novel circulatory system (different from blood vascular or lymphatic) which is distributed throughout an animal's body. We confirmed histologically the presence of liquid flowing channels in BHD (10,11), measured the flow speed in the channels in the BHD on the surfaces of mammalian organs (12), and studied some contents of the liquid such as catecholamine (13) and DNA-containing microcells (14,15). The lymphatic BHD is a part of this network and comes into and out of the vessel walls to be connected to the BHDs on the organ surfaces (7). *Figure 5* demonstrates a case in which a lymphatic BHD comes out of the vessel to enter the surrounding fat tissue, thus becoming a BHD on an organ surface.

Another question is whether the BHD is present in all lymphatic vessels. According to Kim (3), it exists only in large-caliber lymphatic vessels and not in small capillary vessels. This is why the BHD was not observed with highly-sophisticated optical instruments, like a transmission digital microscope (TDM) which can take routine images (whole lymphangion, lymphatic walls, leaflets of valves, and flowing single cells) (16-19). However, TDM is limited to the study of relatively small lymph vessels in the rat mesentery. Hence, developing a similar device to examine large-caliber lymph vessels to study the BHD in detail is highly desirable. The current work provides valuable information for future development of such a device. The spectral transmittance (*Fig. 1*) is useful for detection of a BHD in the lymph vessel. The parallel direction of light relative to the vessel is helpful to minimize the light intensity reflected from the surface of the vessel, and to enhance the contrast. The halogen light source rather than mercury lamp or laser beam was more effective in detection of the BHD. The position and magnification range of the stereomicroscope may provide design parameters of a future system.

Most importantly, concerning the functions of the lymphatic BHC and BHD, we can only find that they are a local generating system of lymphocytes (14,15) whose periodic stages of lymphocyte generation were depicted by Kim (20). As an extended part of the acupuncture meridian system, the lymphatic BHC and BHD may also play important roles in connection with Traditional Chinese Medicine. Finally, it may be worthwhile to investigate the possible role of the lymphatic BHD in connection with metastasis through lymphatic vessels (21).

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REFERENCES

1. Witte, NH, M Ohkuma, M Andrade, et al: Nature's historic gap: The 20th century of lymphology. Lymphology 38 (2006), 157-158.
2. Achen, MG, SA Stacker: Molecular control of lymphatic metastasis. Ann N. Y. Acad. Sci. 1131 (2008), 225-234.
3. Kim, BH: On the Kyungrak system. J. Acad. Med. Sci. D. P. R. Korea 90 (1963), 1-41.
4. Fujiwara, S, SB Yu: "Bonghan Theory" morphological studies. Igakuro Ayumi 60 (1967), 567-577.
5. Lee, BC, KY Baik, HM Johng, et al: Acridine orange staining method to reveal characteristic features of intravascular threadlike structure. Anat. Rec. B New Anat. 278 (2004), 27-30.
6. Lee, BC, SK Kim, KS Soh: Novel anatomic structures in the brain and spinal cord of rabbit that may belong to the Bonghan system of potential acupuncture meridians. J. Acup. Meridian Studies 1 (2008), 29-35.
7. Shin, HS, HM Johng, BC Lee, et al: Feulgen reaction study of novel threadlike structures (Bonghan ducts) on the surfaces of mammalian organs. Anat. Rec. B New Anat. 284 (2005), 35-40.
8. Lee, BC, JS Yoo, KY Baik, et al: Novel threadlike structures (Bonghan Ducts) inside lymphatic vessels of rabbits visualized with a Janus Green B staining method. Anat. Rec. B New Anat. 286 (2005), 1-7.
9. Johng HM, JS Yoo, TJ Yoon, et al: Use of magnetic nanoparticles to visualize threadlike structures inside lymphatic vessels of rats. Evid. Based Complement. Alternat. Med. 4 (2007), 77-82.
10. Lee, C, SK Seol, BC Lee, et al: Alcian Blue staining method to visualize Bonghan threads inside large caliber lymphatic vessels and X-ray microtomography to reveal their microchannels. Lymphat. Res. Biol. 4 (2006), 1-9.
11. Lee, BC, JS Yoo, V Ogay, et al: Electron microscopic study of novel threadlike structures on the surfaces of mammalian organs. Microscopy Res. Tech. 70 (2007), 34-43.
12. Sung, B, MS Kim, BC Lee, et al: Measurement of flow speed in the channels of novel threadlike structures on the surfaces of mammalian organs. Naturwissenschaften 95 (2008), 117-124.
13. Kim, J, V Ogay, BC Lee, et al: Catecholamine producing novel endocrine organ: Bonghan system. Med. Acupunct. 20 (2008), 97-102.
14. Ogay, V, KY Baik, BC Lee, et al: Characterization of DNA-containing granules flowing through the meridian-like system on the internal organs of rabbits. Acup. Electro-Therapeutics Res. Int. J. 31 (2006), 13-31.
15. Kwon, J, KY Baik, BC Lee, et al: Scanning probe microscopy study of microcells from the organ surface Bonghan corpuscle. Appl. Phys. Lett. 90 (2007), 173903.
16. Galanzha, EI, VV Tuchin, VP Zharov: In vivo integrated flow image cytometry and lymph/blood vessels dynamic microscopy. J. Biomed. Opt. 10 (2005), 054018.
17. Galanzha, EI, SS Ulyanov, VV Tuchin, et al: Comparison of lymph and blood flow in microvessels: Coherent optical measurements. Proc. SPIE. 4163 (2000), 94-98.
18. Galanzha, EI, GE Brill, Y Aizu, et al: Speckle and Doppler methods of blood and lymph flow monitoring. SPIE Press, Bellingham, 2002.
19. Galanzha, EI, VV Tuchin, AV Solov'eva, et al: Development of optical diagnostics of microlymphatics at the experimental lymphedema: comparative analysis. J. X-Ray Sci. Technol. 10 (2002), 215–223.
20. Kim, BH: Sanal Theory. J. Acad. Med. Sci. D. P. R. Korea 168 (1965), 5-38.
21. Sleeman, JP: The relationship between tumors and the lymphatics: What more is there to know? Lymphology 37 (2006), 62-68.

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