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Original Article

Exploration of differentiation standard for primo through the histological common point of threadlike structure found in rats and swine

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ABSTRACT

Background: The novel threadlike structure is called the primo structure, and studies are conducted through many different approaches. Although various ways of differentiation are currently used, a standard for differentiation is deemed necessary in order to identify the primo structure based on the overall form of the structure. This study aims to explore the differentiation standard through the histological common point of the threadlike structure of rat and swine by using the hematoxylin and eosin (H&E) staining method commonly used for histological research in various structures.

Methods: An 8-week old Sprague-Dawley rat and a Yorkshire pig weighing 30–40 kg were used. A total of 65 pieces of rat threadlike structure and 100 pieces of swine threadlike structure were collected after the abdomen was cut and opened. The following three different characteristics were confirmed using the H&E staining method for the collected structures: (1) bright cell availability; (2) cavity availability; and (3) nuclei density.

Results: For the rat threadlike structure, the bright cell (70.5%) and nuclei density (92.6%) were mainly observed; in the swine threadlike structure, however, the bright cell (60.6%) and cavity (67.2%) were mainly observed. The bright cell was confirmed to have been observed in the threadlike structures of both rat and swine.

Conclusion: The bright cell is determined to be the common point in the primo structure. However, further research is deemed necessary in the future as to the functions performed by the characteristic shown by the Primo structure.

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1. Introduction

The Bonghan system or threadlike structure, which is called the primo vascular system, has been suggested as the third circulatory system. To study the primo vascular system as a new tissue, specific morphological and molecular biological indices should be suggested. In particular, primo tissues tend to be distributed atypically compared to other tissues; thus, they are less likely to be present in certain locations and are found less frequently. The specific characteristics of primo tissues are seldom identified as well.

Lee et al[1] visualized primo nodes and primo vessels in the sinuses of rat brain using chromium-hematoxylin (Cr–Hx) solution, which was then suggested as a method for primo visualization that can be distinguished from the blood clots developed during surgery. Lim et al[2] suggested that the rat organ-surface primo vessel can be useful in identifying the primo tissues by morphologically observing with Hemacolor staining followed by simple light microscope for a brief time.

Hematoxylin and eosin (H&E) stain is a commonly used staining method to identify the morphological characteristics in various tissues; hematoxylin stains mainly the cell nucleus and cell membranes, whereas eosin stains the cytoplasm, enabling observing the overall morphology of the cell. With this method, the experimental approach is easy, and the database for most human tissues is already established; hence the process of distinguishing various similar tissues is simplified. As such, the histological differentiation of primo tissues using H&E stain should be conducted prior to other studies.

To date, the specific characteristics of the primo tissue were identified by comparing the primo tissue and similar tissues in studies in various animals including mouse,[3] rat,[4] rabbit,[5] canine,[6] and swine.[7] However the comparison of morphological characteristics between primo tissues observed in different species has not been reported. If the same anatomical and histological characteristics are observed in threadlike structures found in various species of animals, they may be the same tissues possibly found in nonexperimented animals and even in humans. Studies on medium and large-sized animals are insufficient compared to those on mice and rats, with a relatively large amount of study results accumulated. Some studies were conducted on swine and canine primo tissues, but the reliability and reproducibility of those studies leave something to be desired because the studies were not followed up due to the lack of laboratory facilities for medium and large-sized animals, aside from the lack of data itself for reference. Therefore, there is a need to accumulate and analyze study results involving medium and large-sized animals for the comparative study of primo tissues in various species.

In this study, rat threadlike structures were histologically analyzed using H&E staining, which is commonly and widely used in histology. On the basis of such analysis, the histological characteristics of swine threadlike structures were compared. Based on these results, the major differentiation criteria for threadlike structures observed in different animals were considered.

2. Methods

2.1. Rat

2.1.1. Experimental animal

About 8–9 weeks old Sprague-Dawley rats ($n = 100$; DBL, Korea) weighing 250–320 g were used in the experiment. Animals were bred at $22 \pm 1\,^{\circ}C$, $55 \pm 10\%$ relative humidity, and 12-hour dark/light cycle. Animals had free access to feed and water during the acclimation period and experimental period, but only water was provided 24 hours before the operation.

2.1.2. Operation and tissue collection

General anesthesia was performed by injecting 20% urethane (Sigma-Aldrich, USA) 1.5 g/kg intramuscularly. The abdomen was cut open by incising along the linea alba to avoid bleeding. During the operation, the drying of the organ surface was prevented by dropping phosphate-buffered saline (PBS, pH7.4) maintained in the water bath at $40\,^{\circ}C$.

The surface of the organ was observed using stereoscopic microscope (SMZ1500, Nikon Japan); milky-white transparent mass or glandular tissues were stained with 0.4% trypan blue (Sigma-Aldrich, USA) and immediately washed with PBS, with the connection of stained features and surrounding tissues subsequently identified before collecting tissues. We used only the vessels of the threadlike structure. The collected tissues were immediately fixed in 10% neutral buffered formalin (NBF) for 24 hours.

2.1.3. Tissue treatment and staining

The fixed tissues were frozen-sectioned at 8 μm thickness and applied to H&E, Masson's trichrome, and immunohistochemistry staining methods. For Masson's trichrome stain, the mesentery was used as positive control and fibrin as negative control. Tissues other than the mesentery were tested by immunohistochemistry. The LYVE1(Abcam) antibody was used for distinguishing from lymphatic vessels, and the CD31(Abcam) antibody, for distinguishing from blood vessels. It was identified using the von Willebrand factor (Abcam) antibody, which shows positive response in the primo tissue.[8]

A total of 65 tissues were collected based on Masson's trichrome stain and the immunohistochemistry results. The basic structures of the cell nucleus and cytoplasm of the collected tissues were observed through H & E stain.

2.1.4. Tissue differentiation

Based on the H&E stain results and references,[9,10] tissues were differentiated by three tissue characteristics. Specifically, primo tissues were differentiated by the presence of cavity, presence of bright cells, and crowding of nuclei. The cavity, which is made of membranes of elastic fiber, was identified by the shape of the holes of epithelial cells (Fig. 1. R-6); the bright cells were detected by observing cells wherein the cytoplasm was not stained by eosin and the nucleus in the center was stained by hematoxylin (Fig. 1. R-2). The degree of crowding nuclei was based on the observation of more than 30 nuclei in a 50 μm × 50 μm area (Fig. 1. R-5).

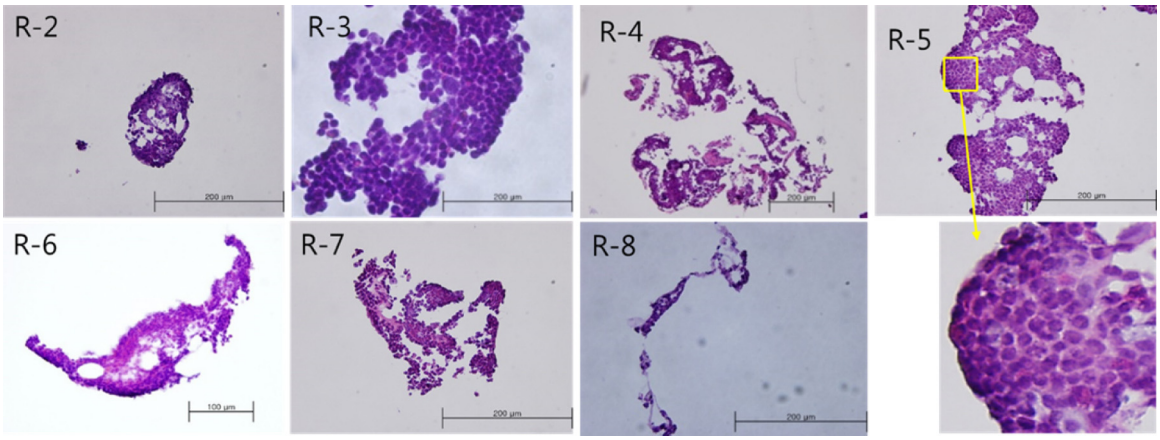

Fig. 1 – H&E stain of rat threadlike structure by type. (R-2) Tissues with bright cell only. (R-3) Tissues with nuclei crowding only. (R-4) Tissues with cavity and bright cell. (R-5) Tissues with cavity, bright cell, and nuclei crowding. (R-6) Tissues with cavity and nuclei crowding. (R-7) Tissues with bright cell and nuclei crowding. (R-8) Tissues with none of the three characteristics. Tissues with cavity only were not observed (R-1). The degree of nuclei crowding was based on the observation of more than 30 nuclei in a 50 μm × 50-μm area (box).

2.2. *Swine*

2.2.1. *Experimental animal*

All experiments were performed according to the Principles of Laboratory Animal Care. Eight healthy Yorkshire pigs each weighing 30–40 kg were used in this study. The animals were bred at 60% relative humidity and 12-hour light/dark cycle. Before the operation, the selected swine was fasted for 12 hours.

2.2.2. *Operation and tissue collection*

The experimental animals were placed on the operating table after intramuscular anesthesia with Rompun (1 mg/kg) and Zoletil (7–10 mg/kg). The operation was performed while monitoring the electrocardiogram, pulse oximetry, end-tidal CO_2, and inspiratory and expiratory gas. Anesthesia was maintained with 50% nitrous oxide in oxygen and 2–3% enflurane end-tidal concentration.

The abdomen was cut open by incising along the linea alba to avoid bleeding into the organs. The threadlike structure on the surface of the swine organ was sprayed with 0.4% trypan blue (Sigma, St. Louis, MO, USA) and immediately washed with PBS; the stained glandular tissues were then observed using stereoscopic microscope (SZX12, Olympus, Japan). Among tissues stained with trypan blue, milky-white tissues separated from other organs and which were not folded were collected. As in the case of the rat, we used only the vessels of the threadlike structure. The collected tissues were immediately fixed in 10% NBF for 24 hours.

2.2.3. *Tissue treatment and staining*

The fixed tissues were frozen-sectioned with the same method used for the rat threadlike structure. The tissues were distinguished from the mesentery through Masson's trichrome stain. The basic structures of the cell nucleus and cytoplasm of the collected tissues different from the mesentery were observed through H&E stain.

2.2.4. *Tissue differentiation*

Tissues were differentiated by applying the criteria for rat threadlike structure tissue differentiation. By analyzing the H&E stain results, tissues were differentiated by characteristics such as presence of cavity, presence of bright cells, and crowding of nuclei.

3. Results

A total of 65 threadlike structures were collected on the surface of rat organs (Fig. 2A). The results of Masson's trichrome stain confirmed that only parts of the rat threadlike structure were stained blue, forming a grain-like pattern. Fibrin cells were observed as a floating pattern and stained bright red compared to the threadlike structure (Fig. 2B and 2C). The threadlike structure of rat showed a positive reaction for anti-von Willebrand factor (anti-vWF). These 65 threadlike structures were identified as neither lymph ducts nor blood vessels using a-LYVE1 and a-CD31 (Fig. 3).

Among the 65 rat threadlike structures, 11 (17%) were excluded from the analysis since none of the 3 characteristics: presence of cavity, presence of bright cells, and crowding of nuclei, was observed in them. Among the 54 rat threadlike structures, 23 (40.8%) were found to have cavity, 38 (70.56%) were found to have bright cells, 50 (92.6%) were found to have nuclei, and 17 (31.5%) were found to have all three characteristics (Figs. 1 and 4A).

A total of 100 tissues were collected from the swine organs (Fig. 5). These tissues were clearly distinguished from the mesentery through Masson's trichrome stain (Fig. 6). The H&E stain results showed that, except 39 (39%) tissues with none of the three characteristics among 100 swine threadlike structure tissues, 41 (67.2%) were found to have cavity, 37 (60.6%) were found to have bright cells, and 8 (13.1%) were found to have nuclei crowding, for a total of 61. Only one (1%) tissue was found to have all three characteristics (Fig. 4B and 7).

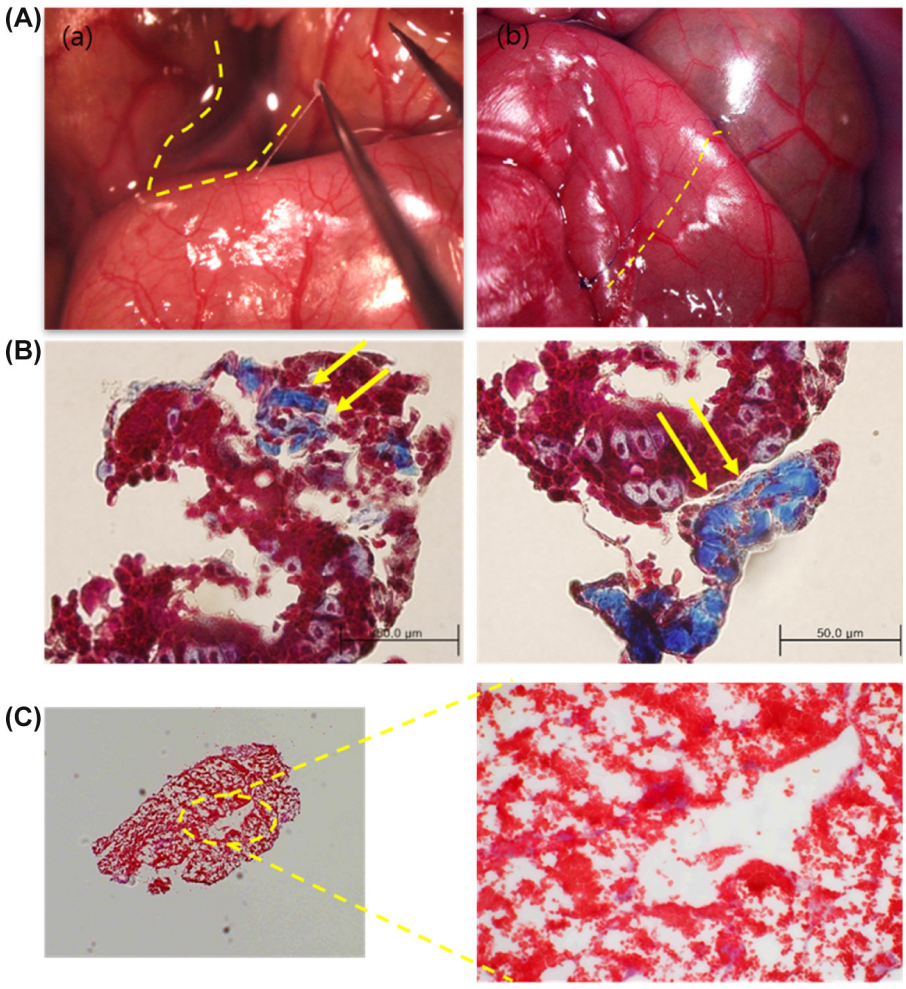

Fig. 2 – Rat threadlike structure on the surface of the abdominal organs. (A) Stereomicroscopic image: (1) organ surface threadlike structure found around the small intestine (dotted line); (2) stained threadlike structure with trypan blue (dotted line). (B, C) Masson's trichrome stain: (B) is the rat threadlike structure, and (C) is the fibrin. In the rat threadlike structure, blue-stained connective tissue was observed in parts of the tissues (arrows). Fibrin was stained bright red compared with the threadlike structure and was consequently distinguished from the threadlike structure (×400). Connective tissue is stained blue, nuclei are stained dark red/purple, and cytoplasm is stained red/pink.

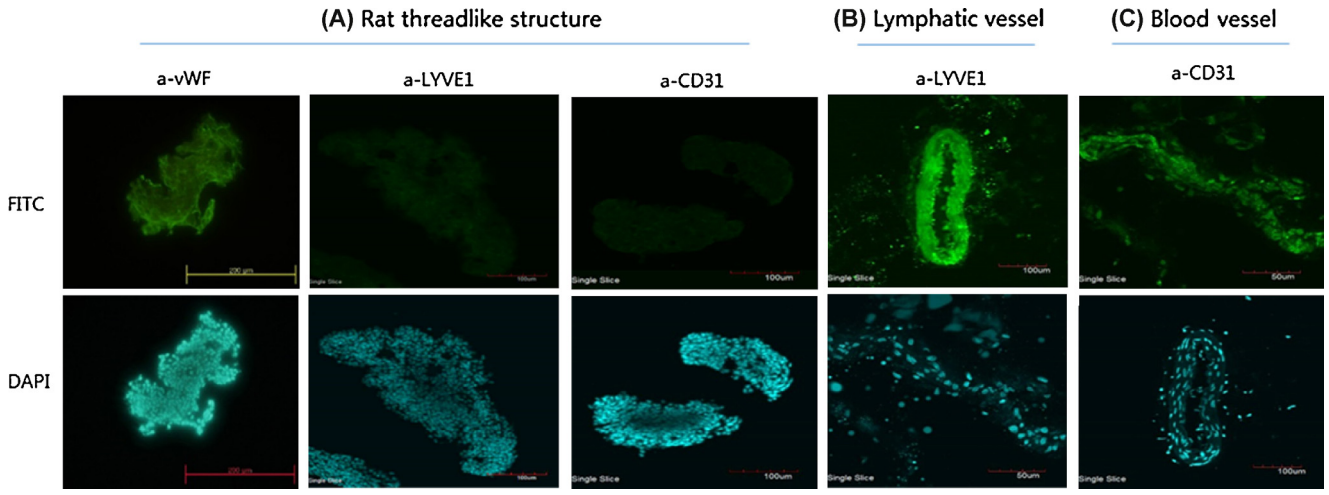

Fig. 3 – Immunohistochemistry of rat threadlike structure. (A) Primo tissue reacted to a-vWF but a-LYVE1 and CD31 did not react. (B) Lymphatic vessels and (C) blood vessels were used as a positive control for LYVE1 and CD31.
DAPI, 4′,6-Diamidino-2-phenylindole; FITC, fluorescein isothiocyanate

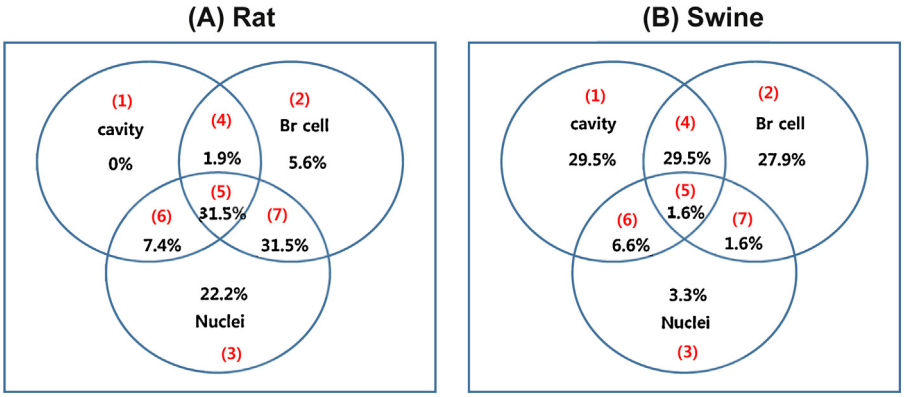

Fig. 4 – Morphological pattern analysis. (A) Rat and (B) swine. A total of 54 rats and 61 swine threadlike structures were analyzed. Tissues with none of the three characteristics were excluded from the analysis. Tissues with cavity, bright cell, and nuclei crowding accounted for 31.5% in rat threadlike structure and 1.6% in swine threadlike structure. Br, bright cell.

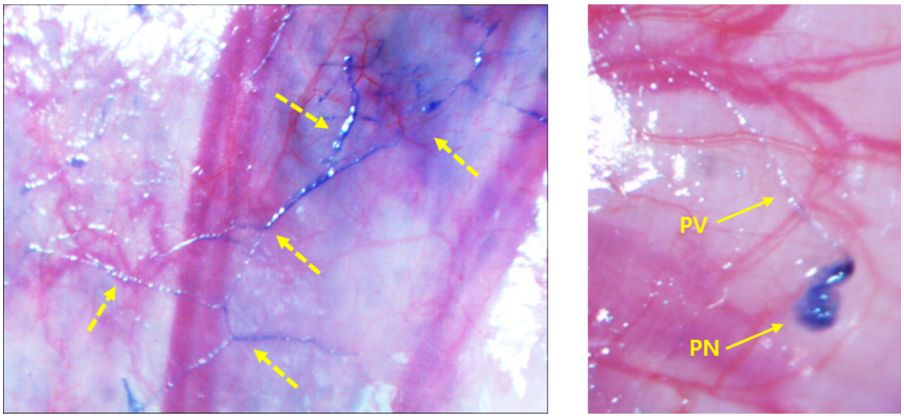

Fig. 5 – Stereoscopic image of threadlike structure of swine. Threadlike structure observed by trypan blue staining on the large intestines. We collected the tissue to form a network structure (dotted arrows) or organization of node (arrow:PN) and vessels (arrow:PV).

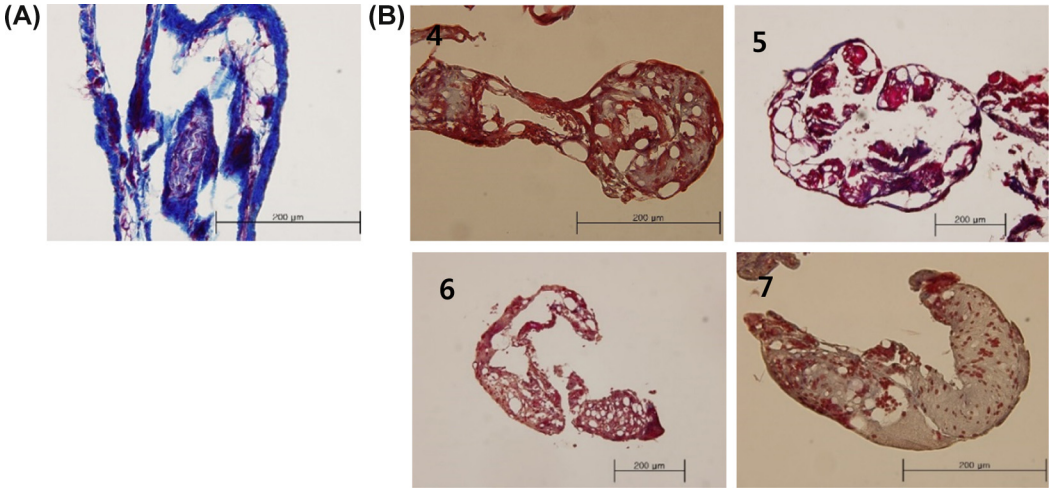

Fig. 6 – Masson's trichrome stain of rat and swine. (A) Swine mesentery used as positive control to be distinguished from the threadlike structure. (B) Rat threadlike structure. Connective tissue is stained in blue; B-4, B-5, B-6, and B-7: Swine threadlike structure. Although the staining feature is different, it is clearly distinguished from connective tissue. Connective tissue is stained blue, nuclei are stained dark red/purple, and cytoplasm is stained red/pink.

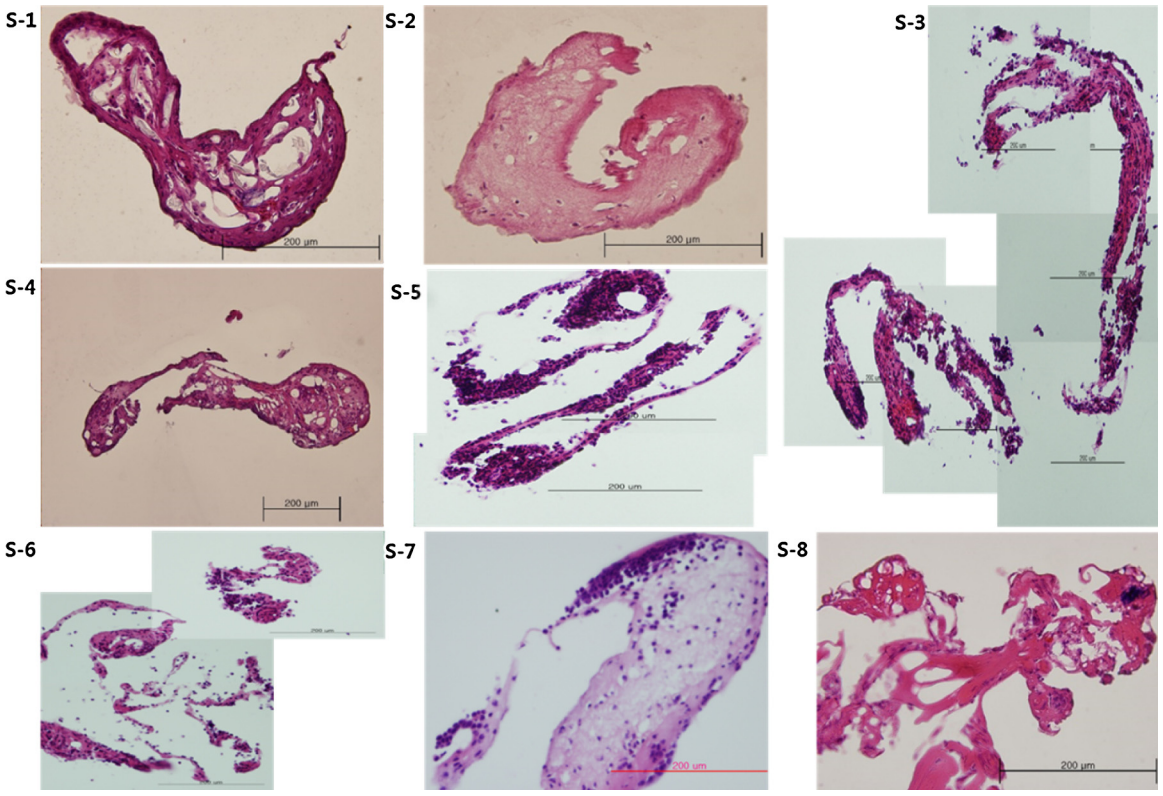

Fig. 7 – H&E stain of swine threadlike structure by type. (S-1) Tissues with cavity only. (S-2) Tissues with bright cell only. (S-3) Tissues with nuclei crowding only. (S-4) Tissues with cavity and bright cell. (S-5) Tissues with cavity, bright cell, and nuclei crowding (S-6) Tissues with cavity and nuclei crowding. (S-7) Tissues with bright cell and nuclei crowding. (S-8) Tissues with none of the three characteristics.

4. Discussion

To date, research on the primo vascular system has started by representing the Bonghan theory announced in the 1960s, expanding to a new area for predicting its functions; the possibilities as a new network system in the body or as stem cells have been reported.[11–13]

Despite approaches from various directions, however, there are obstacles to the development of primo research due to difficulties in collecting samples and controversies over its true nature. Moreover, the histological criteria for verifying the collected samples are not clear; hence the low reliability of research since the criteria for differentiating samples differ by investigator. As such, histological study results should be available to ensure the reliability of primo tissue studies and to conduct more expanded studies.

At present, observations on primo tissues have been made in studies using various animals including mouse, rat, rabbit, canine, and swine experiments. In the case of rat and rabbit, studies on those animals have increased continuously since the early 2000s; however, canine and swine studies were first reported in 2009, and < 10 papers have been published so far.

Primo tissue research involving medium and large-sized animals is a very important step in suggesting the possibility of its presence in humans. However, collecting primo tissues in medium and large-sized animals such as in canine and swine experiments is seldom conducted because most laboratories are not equipped with facilities for medium and large-sized animals. Therefore, there are difficulties in conducting in-depth studies because histological study results involving medium and large-sized animals have not accumulated, with the differentiation criteria for primo tissues and reference data lacking.

This study was designed to provide one of the criteria for primo differentiation by dividing rat and swine threadlike structures by type and then finding the common histological features. Since the differentiation criteria for primo tissues have not been established, this study used the term “threadlike structure” instead of “primo tissue.”

The criteria were established on the basis of characteristics identifiable by H&E stain, among characteristics identified in the paper by Kim.[9,10] According to Kim: (1) basophile granules and small nucleus-like structures (crowded nuclei) were often observed in the small tubule of the Bonghan duct; (2) bright cells were detected in the Bonghan corpuscles; and (3) the cavity enclosed by endothelial cells was reported by observing several bundles of structures.

A total of 65 rat threadlike structures were analyzed for histological characteristics using H&E stain; 17 (26%) showed all three characteristics and 11 (17%) had none of the three characteristics. Tissues with none of the three characteristics described in the paper by Kim were excluded from the analysis because they were assumed not to be primo tissues. Among

tissues used in the analysis (54 tissues), 40.1% showed the cavity, 71.1% had bright cells, and 91.6% showed nuclei crowding (Fig. 4A).

In swine threadlike structures, only one (1%) showed all three characteristics and 39 (35%) had none of the three characteristics. As in the case of rat threadlike structures, tissues with none of the three characteristics were excluded from the analysis. Among tissues used in the analysis (61 tissues), 62.3% showed the cavity, 61% had bright cells, and nuclei 13.1% showed crowding (Fig. 4B).

Rat and swine threadlike structures differed in terms of "crowded nuclei" with 92.6% and 13.1%, respectively, and "presence of cavity" with 40.8% and 67.2%, respectively. The "presence of bright cells" was observed in over 60% of tissues. Thus, bright cells were commonly observed in rat and swine threadlike structures by H&E stain. In the current study, the bright cell was identified as a very important index for rat and swine threadlike structures. Kim[9] reported that there are cells with bright cytoplasm with lots of chromaffin granules. It is considered that bright cells are matched to such characteristics. Bright cell that was observed in this study is characterized by the size 10∼12 μm, round shape and cytoplasm not stained with eosin. Although neutrophils and bright cells share some same properties, bright cells are mononuclear and part of the threadlike structure with no mobility, unlike polymorphonuclear and highly mobile neutrophils. More studies should be continued in the future to clearly find out the distinct and unique characteristics of the bright cell.

The threadlike structures observed in rat and swine showed different features in addition to the common feature of bright cell. The difference in rat and swine threadlike structures is deemed to be related to animal size or weeks of age. In other words, the threadlike structure observed in small-sized animals is assumed to have relatively smaller-sized tissues, lower ratio of cavity, and higher degree of nuclei crowding; on the other hand, the swine threadlike structure had bigger tissues, wider ranges of cavity, and lower degrees of nuclei crowding. Moreover, such can be attributed to the difference in specific characteristics among animals, which has yet to be identified. Tissues with the same function can differ in their morphological features depending on the animals. Finally, morphologically different results can be obtained because of the methodological differences in anesthesia and operational processes for small-sized rats and medium-sized swine.

The differences between the rat and swine groups are too big to be used to predict results from other animal groups on the basis of the threadlike structures observed. Therefore, more distinct tendencies could be predicted if more study results are accumulated from other in-between sized animal groups.

Definite differentiation criteria for primo tissues may not be suggested based on the results of this study. Nonetheless, experiments using at least two differentiation criteria taking into account the histological characteristics above should be conducted to minimize error in the primo study. This study is expected to provide the guidelines for differentiating primo tissues through the histological comparison of rat and swine threadlike structures for other researchers and contribute to the preparation of differentiation criteria for primo tissues.

More in-depth studies are required in the future to determine the relationship between the characteristics of primo tissues and related functions by expanding the research efforts on threadlike structures. Before that, however, the term "primo vascular system" should be used clearly instead of "threadlike structure" by establishing the differentiation criteria for primo tissues.

Conflicts of interest

All authors have no conflicts of interest to declare.

Acknowledgments

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REFERENCES

1. Lee HS, Park WH, Je AR, Kwon HS, Lee BC. Evidence for novel structures (primo vessels and primo nodes) floating in the venous sinuses of rat brains. *Neurosci Lett* 2012;522:98–102.
2. Lim CJ, Yoo JH, Kim YB, Lee SY, Ryu PD. Gross Morphological Features of the Organ Surface Primo-Vascular System Revealed by Hemacolor Staining. *eCAM* 2013:350815.
3. Hong MY, Park SS, Do HK, Jhon GJ, Suh MA, Lee YM. Study of the Primo-Vascular system and Location-Dependent Oxygen Levels for a Mouse Embryo. *J Nanosci Nanotechnol* 2012;12:5168–72.
4. Jung SJ, Bae KH, Nam MH, Kwon HM, Song YK, Soh KS. Primo Vascular system Floating in Lymph Ducts of rats. *J Acupunct Meridian Stud* 2013;6:306–18.
5. Noh YI, Rho MS, Yoo YM, Jung SJ, Lee SS. Isolation and morphological features of primo vessels in rabbit lymph vessels. *J Acupunct Meridian Stud* 2012;5:201–5.
6. Zhao FJ, Coh KS, Zhou Q, Dong B, Yu WH. Study of novel Threadlike structures on the intestinal fascia of dogs. *J Acupunct Meridian Stud* 2012;4:98–101.
7. Bae KH, Park SH, Lee BC, Nam MH, Yoon JW, Kwon HM, et al. Novel threadlike structures may be present on the large animal organ surface: Evidence in swine model. *eCAM* 2013:758763.
8. Kwon BS, Ha CM, Yu SS, Lee BC, Ro JY, Hwang SH. Microscopic nodes and ducts inside lymphatics and on the surface of internal organs are rich in granulocytes and secretory granules. *Cytokine* 2012;60:587–92.
9. Kim BH. The Kyungrak system. *J Jo Sun Med* 1965:1–38.
10. Kim BH. On the Kyungrak System. *J Acad Med Sci. DPR Korea* 1963;12:1–35.
11. Yeon SH, Kwon OS, Lee SB, Cho SJ, Choi KH, Lee SH, et al. Histological study of the primo vascular system on the falciform ligament. *Korea journal of oriental medicine* 2012;18:131–7.
12. Lee BC, Kim HB, Sung B, Kim KW, Sohn J, Son B, et al. Network of endocardial vessels. *Cardiology* 2011;118:1–7.
13. Park ES, Lee JH, Kim WJ, Heo JB, Shin DM, Lem CH. Expression of stem cell markers in primo vessel of rat. *eCAM* 2013:438079.