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RESEARCH ARTICLE

Observation of Coiled Blood Plexus in Rat Skin with Diffusive Light Illumination

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Abstract
Blood plexuses are characteristic anatomical features of acupuncture points (APs). We developed an optical technique using diffusive light illumination to increase the brightened area of skin for observation of the blood plexuses in skin. We found that the blood plexuses were coiled blood vessels which came out of the perforations in the fascia of muscle. The coiled vessels could be straightened by stretching the skin. We observed a series of blood plexuses at the putative APs along the left and right kidney meridian lines in the abdominal skin of rats. In addition, the locations of the plexuses on the muscle fascia were just above the putative acupuncture muscle channels along the kidney meridians. Furthermore, immunohistochemical analysis of the skin specimens of the plexuses revealed its neurovascular bundle nature as expected from known anatomical features of the APs.

1. Introduction

Studies aimed at understanding the acupuncture point (AP) system from a 'Western' perspective searched for histological features that could differentiate APs from surrounding tissue. Bong Han Kim was the only one who claimed to find specific corpuscular structures at the acupoints [1], which has not been confirmed yet. Nevertheless, several authors suggested some characteristic features of the acupoints without corpuscle like structure.

Heine described that there existed an accompanying composite of a blood vessel and a nerve within a sheet of loose connective mesenchym perforating the superficial fascia that separates the subcutaneous from muscle tissue [2–4]. This blood vessel-nerve complex at the acupoints was confirmed by other researchers [5–9]. Acupoints have also been described as neuromuscular bundles [10,11], veins perforating fascia [12], neuromuscular attachments [13–16], and various types of sensory nerve endings [17–19]. A gathering of leucocytes and mast cells around a blood plexus [20,21], and an abundance of loose connective tissue and a significant concentration of acid mucopolysaccharides [22] were other features of acupoints.

In the present work we investigated the abdominal skin of rats to search for the neurovascular plexus as characteristic features of acupoints. As expected from human chart of acupoints we examined the corresponding area of kidney meridian lines (K-line) and stomach meridian lines (ST-line) and

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found set of blood vessel-nerve complexes located along the K- and ST-lines. In addition, we introduced an optical technique to observe the blood plexus more easily without surgery. This diffusive light illumination technique can be applied for identification of the APs in skin if the blood-plexus are proven to be the classical acupoints.

The blood plexus had a distinguishable 'head' part in the dermis layer, relatively long blood vessel, and the 'tail' part that enters the muscle layer. In its natural pose the blood vessel formed a 'coil', and beame straight line when the skin was stretched. This distinctive feature was clearly observed by using diffusive illumination technique. In the last section we discuss the immunohistochemical study of the plexus with the purpose to compare them with the properties of Bonghan corpuscles (BHCs).

2. Materials and Methods

2.1. Animal preparation

Six- to 12-week-old female Sprague-Dawley rats were obtained from Jung Ang Laboratory Animal Company (Seoul, Korea). The animals were maintained on a 12-h light/dark cycle in temperature and humidity controlled animal room. The rats had *ad-libitum* access to food and water, and the procedures involving the animals and their care were in full compliance with current international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996). The animals were anesthetized with Rompun (50 mg/kg) and then the abdominal area was carefully shaved without damaging of skin layer.

2.2. Determination of APs

The locations of the APs on conception vessel meridian (CV), kidney meridian (K) and stomach meridian (ST) were determined by an acupuncture-expert using an acupoint/meridian map of animals and humans [23,24]. Foregoing acupoints were chosen for our investigation based on the following reasons: (1) the acupoints can be easily determined on the body; (2) the acupoints and non-acupoints located on the abdomen can be isolated simultaneously without separation skin layer from abdominal muscle wall, thereby maintaining the structural integrity of tissue explants; (3) abdominal skin layer is more thinner than skin on dorsal area of the rat. Therefore, observation of blood vessel plexuses and bundles in the abdominal skin can be easily performed by using a method of diffusive light illumination. In order to determine acupoints and meridians we used proportional measure system where the relative unit is the cun. To calculate the cun for the rat we first determined the anatomical location for CV8 (umbilicus) and CV16 (the fifth intercostal space, on the xiphosternal synchondrosis). A distance between them was about 58 mm. Then we determined CV12 on the ventral midline of the abdomen which was located at the midpoint between CV8 and CV16. Hence, the distance from CV8 to CV12 was 29 mm that relatively equals to 4 cun. Having divided 29/4 we found that 1 cun = 7.25 mm. Thus using this value (1 cun = 7.25 mm) we determined the location of the K-meridian and the distance between APs.

2.3. Imaging of blood plexuses in the skin by diffusive illumination method

To visualize blood plexuses in the skin a simple optical system was used as shown in Figure 1. The optical system is consisted of a broadband halogen light source (HLS150W, Xenosys, Korea), optical fiber 3 mm in diameter (Xenosys, Korea) and a stereomicroscope (SZ-X10, Olympus, Japan) with integrated digital camera (DP70, Olympus, Japan). Prior to imaging the rat skin was pretreated by 75% glycerol solution for 20 minutes at room temperature to improve tissue transparency. In addition to increase light penetration depth during diffusive illumination, 0.2 mL of 0.9% physiological saline was subcutaneously injected into acupoint areas. *In vivo* imaging was performed by directing the diffusive light illumination at the acupoint area and detecting of blood vessels by using the stereomicroscope. In this study a use of diffusive illumination provided many advantages such as raised brightness of illumination from inside, sharpened contrast of the blood vessels in different skin layers, and improved details of blood plexuses and bundles in the tissue.

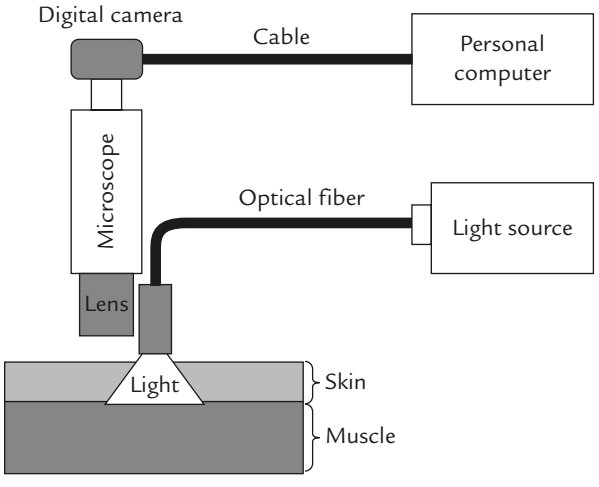

Figure 1 Schematic illustration of the optical system for observation of blood vessels in the skin.

2.4. Anatomical observation

Under deep anesthesia an incision along K-meridians was made and then the edges of skin were lifted by the forceps to carry out the anatomical observation of the neurovascular bundles (NBs). After stereomicroscopic examination abdominal skin was removed from the abdomen and then a general view of the distribution of NBs was captured by a digital camera.

2.5. Immunohistochemistry

Tissue explants including skin layer, subcutaneous connective tissue and abdominal muscle wall were excised from the rats and fixed in a fixative consisting of 0.5% paraformaldehyde (Sigma, USA) and 15% (v/v) saturated picric acid (Sigma, USA) in 0.1 M sodium phosphate buffer (SPB, pH 7.0) overnight at 4°C. Prior to sectioning, the tissue samples were infiltrated in O.C.T. compound (Sakura Finetek, USA) and frozen in isopentane at the temperature of liquid nitrogen. The frozen samples were placed on cryostat and then sectioned by using a crytome (Microm Lab. HM 505E, Germany) at 100 μm. Tissue sections were rinsed in PBS for 15 minutes to remove O.C.T. embedding material and incubated in a 3% sodium deoxycholate solution (Fluka, Italy) for 4 hours at room temperature under mild agitation in 24-well plate (Falcon, USA). The specimens were rinsed twice with distilled water and then with PBS for three times 1 hour each. The sections were incubated in 10% normal goat serum (NGS) (Chemicon Inc., USA), overnight at 4°C, then 1 day at 4°C with rabbit anti-neurofilament 150 kD polyclonal antibody (1:200, Chemicon Inc., USA), mouse anti-RECA-1 (1:200, Abcam, UK), rabbit polyclonal anti-rat LYVE-1 (15 μg/mL, Abcam, USA) and mouse anti-rat CD31 (1:50, BD Pharmingen, USA). After washing with PBS, the specimens were incubated with the appropriate secondary antibodies for 1 day at 4°C: Alexa Flour 555 goat anti-rabbit Ig G (H+L) (1:500) and Alexa Flour 488 goat anti-mouse Ig G (H+L) (1:500), (all from Invitrogen, USA). All antibodies were diluted in 10% NGS containing 0.1% NaN_3 (pH 7.4). After each incubation with antibodies, sections were washed in three changes of PBS. Tissue sections were stained by DNA specific dye (DAPI), mounted in anti-fading medium and observed with both a stereomicroscope (MVX-10, Olympus, Japan) and a confocal laser scanning microscope (LSM 510, Carl Zeiss, Germany).

2.6. Staining dye injection

Chrome-hematoxylin was useful for visualization under light stereomicroscopy. Under a stereomicroscope, we injected 20–30 μL of chrome-hematoxylin into body muscle layer of rat skin by using insulin syringe (31 gauge, 0.3 mL, Becton, Dickinson and Company, USA). Immediately after injection, we observed a relatively long trace of staining dye in body muscle layer.

3. Results

3.1. Anatomical observation

According to our calculations 1 cun for the rat body was equal to 7.25 mm (see Materials and Methods). Thus, using given value we determined the locations of APs and meridians on the abdominal skin of the rats. For example, K- and ST-meridians were located 3.62 mm (0.5 cun) and 14.5 mm (2 cun) lateral to the midline of the epigastrium, respectively. The distance between APs was 7.25 mm. After mapping APs on the abdominal skin we examined whether marked APs on the skin correspond to anatomical locations of the NBs and plexuses. Anatomical observations revealed that only 7 out of 11 pairs of APs on K-meridian were related to anatomical distribution of the NBs perforating the superficial fascia and abdominal muscle wall (Figure 2F). It was observed that APs K14, K15 and K16 were exactly associated with the locations of the NBs (Figure 2A), whereas the points K17 to K21 were located near to the NBs (Figure 3B). However, under the points K11 to K13 no NBs was observed. Similar data was obtained from microscopic observation of ST-meridian and the points ST20 to ST30. It was found that only two points ST20 and ST21 were related to the anatomical location of the NBs as shown in Figure 3B. Morphometric analysis showed that NBs were located about 4 mm lateral to the middle of the epigastrium (Figure 2D). It approximately corresponded to the location of KI-meridian. The distance between the NBs located on K- and ST-meridian varied from 7 to 9 mm depending on individual animal (Figure 2C).

3.2. Imaging of blood vessels with diffusive illumination method

To visualize NBs or plexuses at the acupoints non-invasive diffusive illumination method was applied. Diffusive illumination method was the essential method that enhanced the study of the blood plexus under skin in a number of manners: raised the brightness of the area under observation, sharpened the contrast between different layers of blood vessels, and improved the details of morphological structures observable with naked eyes. The diffusive illumination method with no saline injection had a relatively small area under illumination (Figure 4A). Approximated direct illumination diameter was

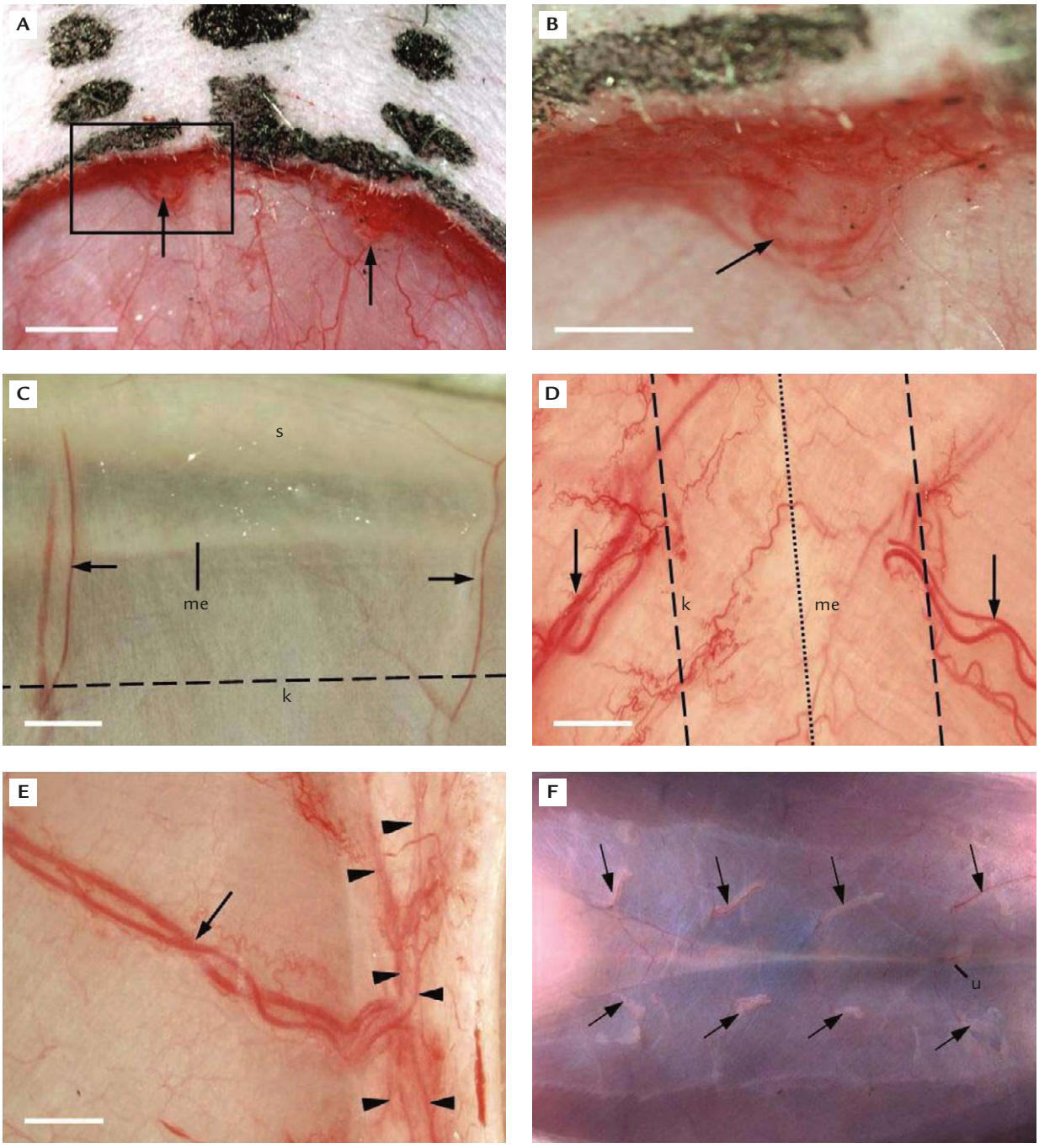

Figure 2 Morphology and anatomical distribution of the neurovascular bundles (NBs) and plexuses at K-meridians. **(**A) Two coiled NBs (arrow) at acupuncture points K15 are seen in subcutaneous connective tissue layer. **(**B) Magnified image of the rectangular area in Figure 2A shows coiled morphology of the NB (arrow). **(**C) NBs (arrow) in the stretched state are located at a distance of 9 mm from each other. (s) – skin; (me) – middle epigastrium; (k) – kidney meridian. (D) NBs (arrow) are symmetrically located on both sides of K-meridians. A distance from the middle of the epigastrium (me) to NB is about 4 mm. (E) NB (arrow) forms several branches of blood vessels (arrowhead) in the subcutaneous layer. **(**F) Large view of the anatomical distribution of the NBs (arrow) along K-meridians on the abdominal side. (u) – umbilicus. Scale bars = 2 mm in A, C, D and E, 1 mm in B.

about 1 cm and diffusive illumination diameter was about 2 cm. Diffusive light illumination system with no saline injection showed the 'tail' of the blood plexus, the location of the coiled blood plexus and displayed the shape of the coil (Figure 4C). Moreover, the location of the 'head' of the plexus and the diversification of the blood plexuses into several vessels could be seen with naked eyes. However, detailed blood plexus morphology could not be imaged, but only the blood vessels on the dermal layer were

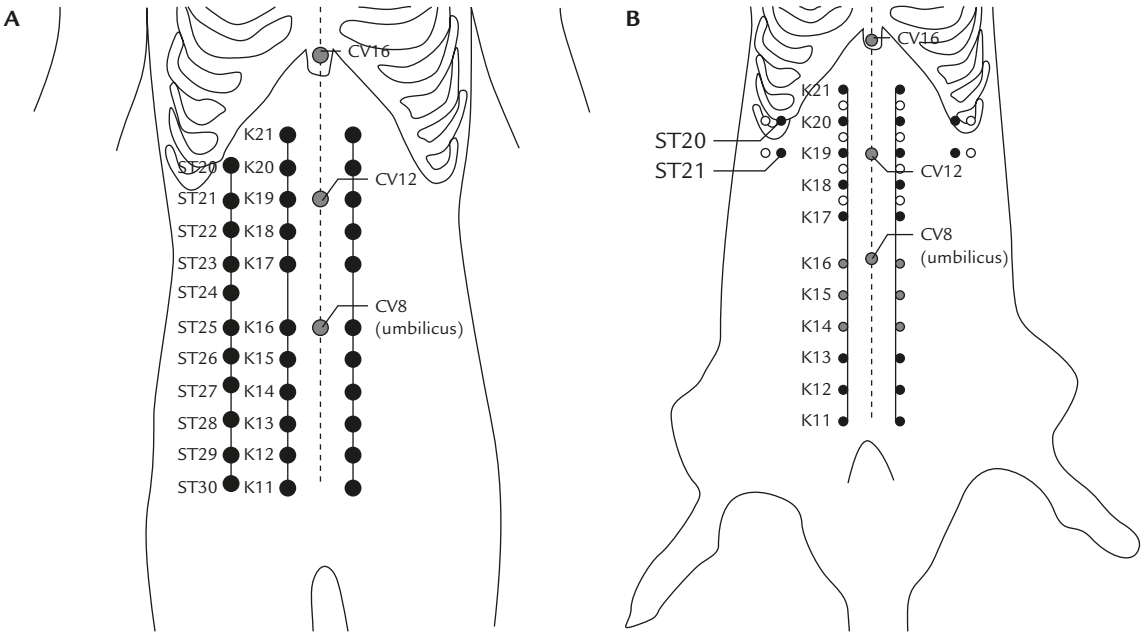

Figure 3 Relationship of the neurovascular bundles (NBs) and the acupuncture points (APs) on Kidney and Stomach meridian in a rat. (A) Schematic illustration showing the locations of APs in a human. (B) Distribution of the NBs and mapped APs on K- and ST-meridians in the rat. Note that the points K14 to K16 (grey circle) exactly match to the locations of the NBs. The points K17 to K21 and ST20, ST21 (black circle) are located near the NBs (white circle).

distinctively recognized on the images taken by the microscope and the digital camera. Thus, these difficulties led us to create the new method of saline injection into the rats.

In order to improve the quality of images, saline was injected into the rat in two different layers: subcutaneous layer or intra-peritoneum. Subcutaneous injection of saline amplified the brightness of the specific injection area and the sharpened the details of structure of the blood plexus (Figure 4B, D). The skin was inflated forming a shape of a half sphere (size is depended on the amount of saline being injected) around the injection point. This helped to brighten up that specific injection area, where the saline was injected and stayed. 0.2 mL of saline injection improved the illumination diameter to 2.0 cm of direct illumination and 3.7 cm of diffusive illumination (Figure 4B). With naked eyes, blood vessels on the surface of the skin, the stretched blood plexus and the 'head' of the blood plexus could be distinguished even more clearly. The improvements were also evident under the microscope. As seen in Figure 4D, the location of the 'head' of the plexus and the diversification of the blood plexuses into several vessels could be imaged by a microscope. Furthermore, the contrast between blood vessels on the skin surface and the stretched blood plexus heading inward to the muscle layer could be improved. However, coiled shape of the blood plexus and the 'tail' part of the blood plexus could not be seen. The inflated skin increased the distance between the skin layer and the muscle layer, and this caused the stretching of the blood plexus and difficulties in viewing the 'tail' part of the blood plexus, which became further away from the dermis.

Saline was also injected into the intra-peritoneum. Compared to the illumination area with no injection, the intra-peritoneal injection brought improvements in illumination intensity for a large area. 0.15 mL of saline in intra-peritoneum injection had a direct illumination diameter of 2.4 cm and a diffusive illumination diameter of 5.5 cm. Results from intra-peritoneum injection were similar to the injection into subcutaneous layer. With naked eyes, blood vessels on the skin surface, the stretched blood plexus and the head of the blood plexus could be distinguished (Figure 5). Blood plexus were not visible in the coiled shape and the 'tail' was not seen. However, a noticeable difference was present in the quality of image taken. The head of the blood plexus and also the contrast of the blood vessels on skin surface and the blood plexus leading into the muscle layer were vividly imaged as seen in Figure 5.

3.3. Blood plexus and acupuncture muscle fascicle

The tail part of blood vessels in the plexus entered the body muscle layer through a perforation in the fascia (Figure 6A) which had a criss-cross structure of fibers. As chrome-hematoxylin was injected into the muscle fascicle running along the K-line, the staining dye flowed out through the perforation (Figure 6B).

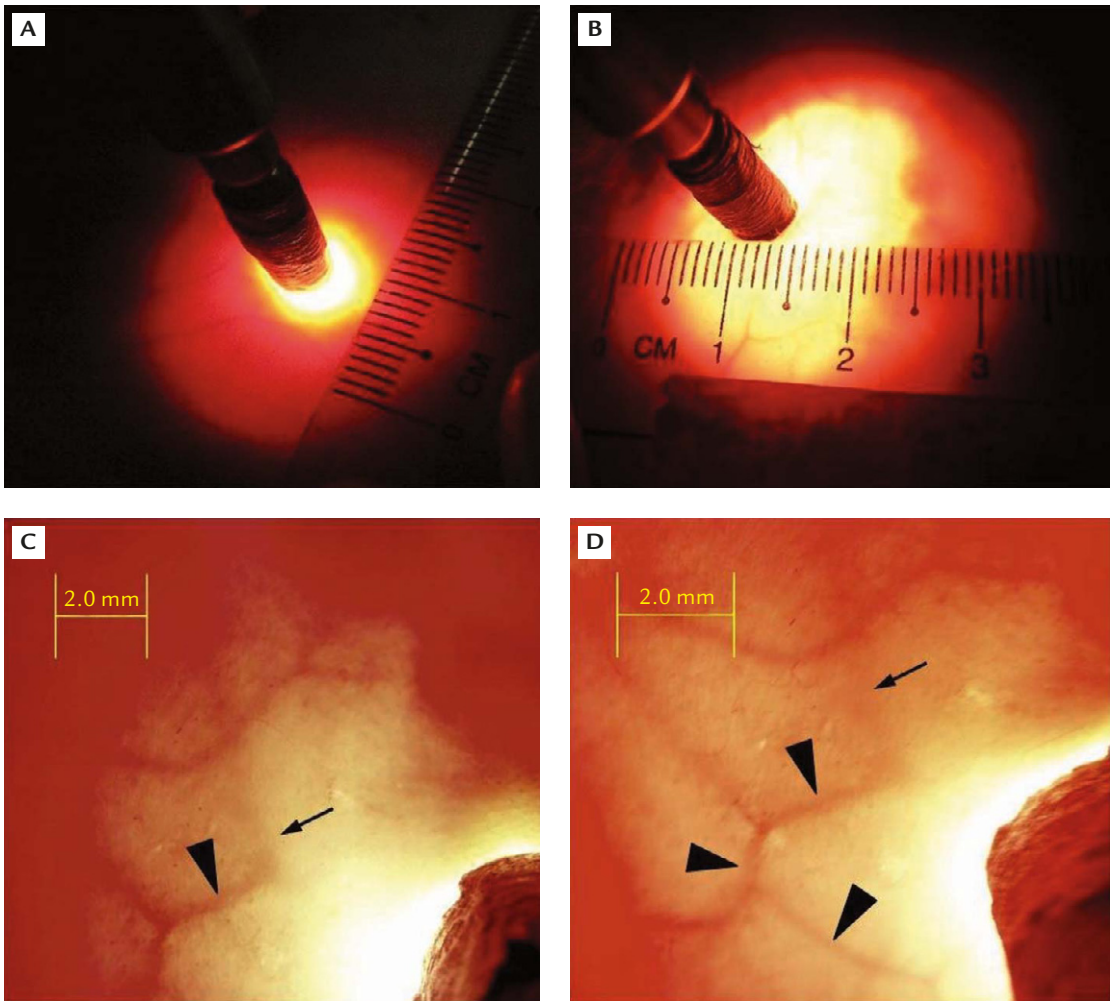

Figure 4 Imaging of blood vessel bundles at acupuncture points with diffusive illumination method. (A, C) without and (B, D) with application of saline injection. Arrow and arrowhead indicate blood vessel bundle and branches of blood vessels respectively.

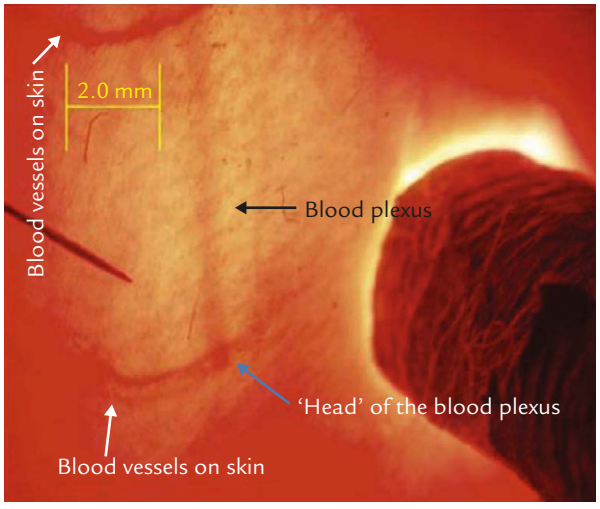

Figure 5 Intra-peritoneal saline injection. Blood vessels on the skin surface and the blood plexus leading into the muscle layer were imaged in vivid contrast. 'Head' of the blood plexus and the diversification of blood vessels on the head was also imaged with improved contrast.

4. Discussion

Anatomical characteristic features of APs found by various researchers including Heine [2–4] were related to blood vessel and nerve plexuses in the subcutaneous region [5–19]. Based upon these earlier works we hypothesized that there will be systematic distribution of neurovascular plexuses along the left and right kidney meridians (K-line), and the left and right stomach meridians (ST-line). In this work we confirmed this hypothesis by observing a series of neurovascular plexuses as expected. The perforation in the muscle fascia (Figure 6A, B) through which the blood vessels entered is especially in good agreement with Heine's description on human acupoints [2–4]. A schematic illustration of morphometric scales of the plexuses at putative acupoints are presented in Figure 7. The K-lines were approximately 4 mm off from the midline, that is, the conception vessel (CV), and the ST-lines were about 2 cm from the CV line. The locations in the transversal sections of the

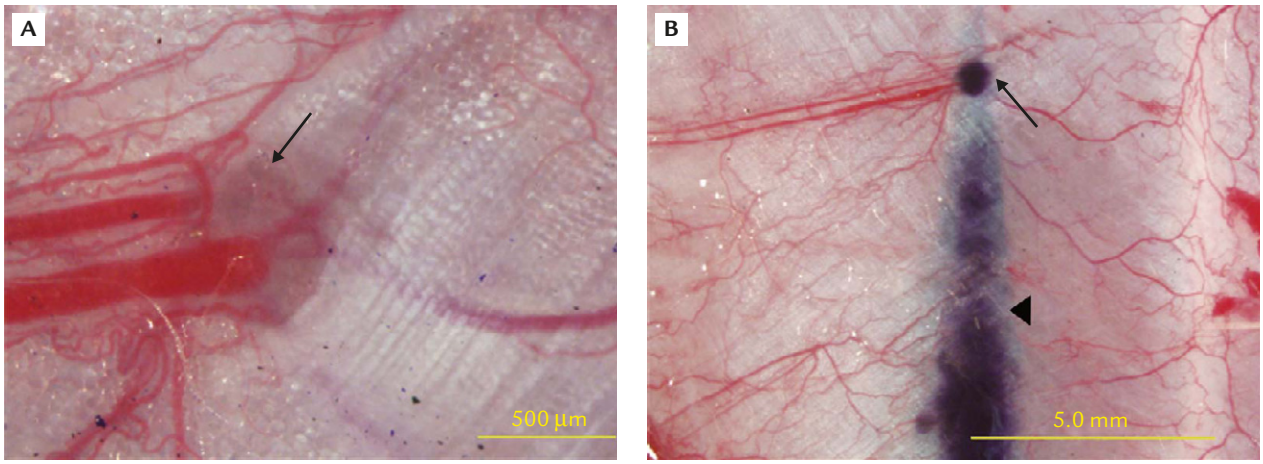

Figure 6 The blood plexus and the acupuncture muscle fascicle. (A) The tail part of the blood vessels entered the body muscle layer through the perforation (arrow) in the fascia. The fascia had a criss-cross structure of fibers. (B) The staining dye chrome-hematoxylin flowed in the channel of muscle fascicle (arrowhead) along the K-line, and the dye flowed out through the perforation (arrow).

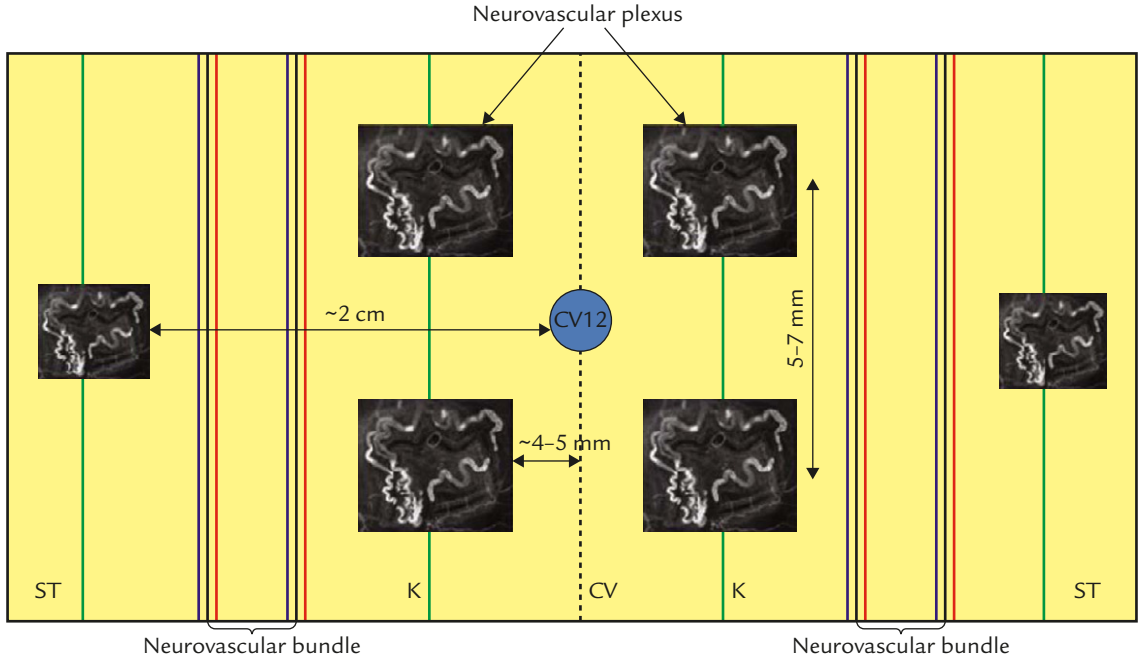

Figure 7 A schematic illustration of neurovascular plexuses of coiled blood vessels along the kidney meridian (K) and stomach meridian (ST) which are 4–5 mm and 2 cm off from the conception vessel (CV) of a rat. Notice that other neurovascular bundles between the K and ST are straight and not coiled at all.

K-lines are shown in Figure 8. The neurovascular plexuses were located in the hypodermis layer as shown in the rectangle of Figure 8A. Immunohistochemical analysis using CD31 (for blood capillaries), NF150 kD (for nerves), and LYVE-1 (for lymph vessels) showed the plexuses were abundant with coiled structures (Figure 8B and C).

In order to investigate the neurovascular plexuses in more detail we made immunofluorescence analysis with RECA-1 and anti-neurofilament 150 kDa visualizing nerve fibers and blood vessels, respectively. The morphometric description is shown in Figure 9A. The center of the plexus was 4 mm off from the CV-line, and the diameter was 2 mm, and the distance between two adjacent plexuses was 5 mm. The coiled blood vessels, and the nerves are shown in Figure 9B.

Transversal sectional views of a typical neurovascular plexus at ST-line with immunohistochemical staining 150 kDa and RECA-1 are shown in Figure 10A, B for the blood vessels, and nerves, respectively. The circled region is the putative acupoint, and its combined and magnified image is shown in Figure 10C. In order to study cellular distribution of the plexus we used DAPI staining that specifically revealed nuclei. The plexus is shown by the broken line of the

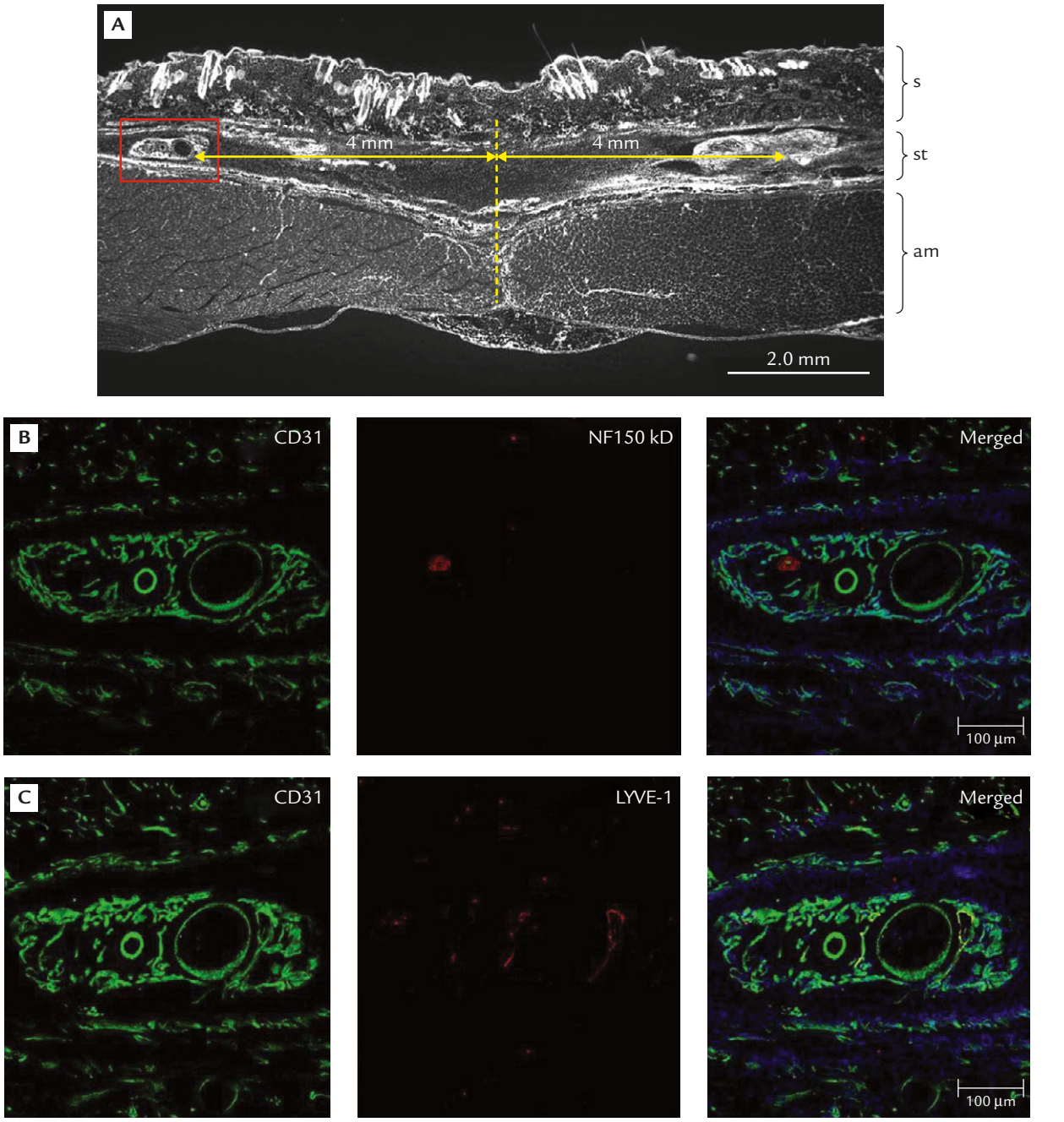

Figure 8 Anatomical location and immunofluorescence imaging of the neurovascular plexuses in the rat abdomen. (A) Histological thick section of tissue explants including the skin (s), subcutaneous tissue layer (st) and abdominal wall muscle (am) shows that neurovascular plexuses are located under skin about 4mm away from the middle epigastrium or conception vessel (dashed line). (B and C) Immunofluorescence imaging of the rectangular area of A showing the distribution of the nerve fibers, blood and lymphatic vessels in the neurovascular plexus. Cell nuclei (blue) are stained by DAPI.

ellipse, and there were high concentration of nuclei in the plexus (Figure 11). It is located about 1 mm below from the skin surface and enclosed by muscular layers.

The reason we present the immunohistochemical data of the neurovascular plexuses in the discussion rather than the result section is the uncertainty in identifying them as either the putative acupoints or the BHCs [1]. According to Kim, a corpuscle is located in the dermis, and there are many blood capillaries and nerve endings in it. The corpuscle is connected by the bundle of blood vessel and Bonghan ducts (BHD) that runs into the muscle layer. Therefore, Kim's description on BHC and duct is consistent with the anatomical description of human acupoints by Heine and others [2–19]. Our observation of the neurovascular plexuses in rats is also in agreement with Kim's and others.

Yet, we cannot conclude our observation is the confirmation of the BHC for the following reasons.

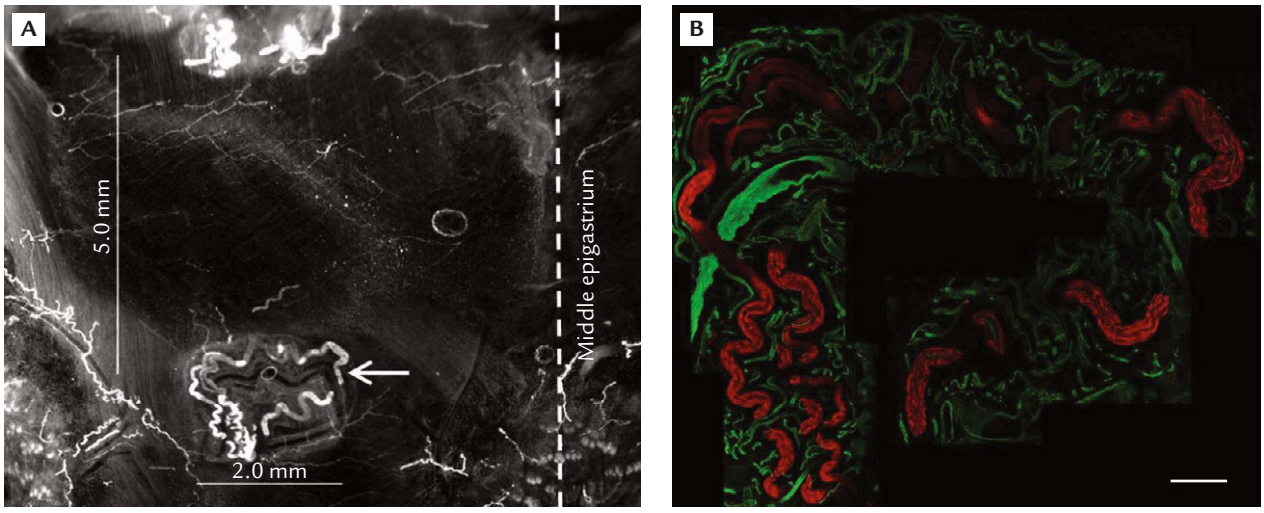

Figure 9 Distribution and morphometric characteristics of the neurovascular plexuses. (A) Stereomicroscopic image (Z-plane section) showing two neurovascular plexuses which are located about 4 mm off from the middle epigastrium (dashed line). The distance between the plexuses is about 5 mm. A diameter of the plexus is about 2 mm. (B) Confocal image of the neurovascular plexus indicated by the arrow in A showing a complex network of blood vessels and capillaries (green) around nerve fibers (red). Blood vessels and nerve fibers were stained with RECA-1 and NF-150 kD respectively. Scale bar, 200 μm.

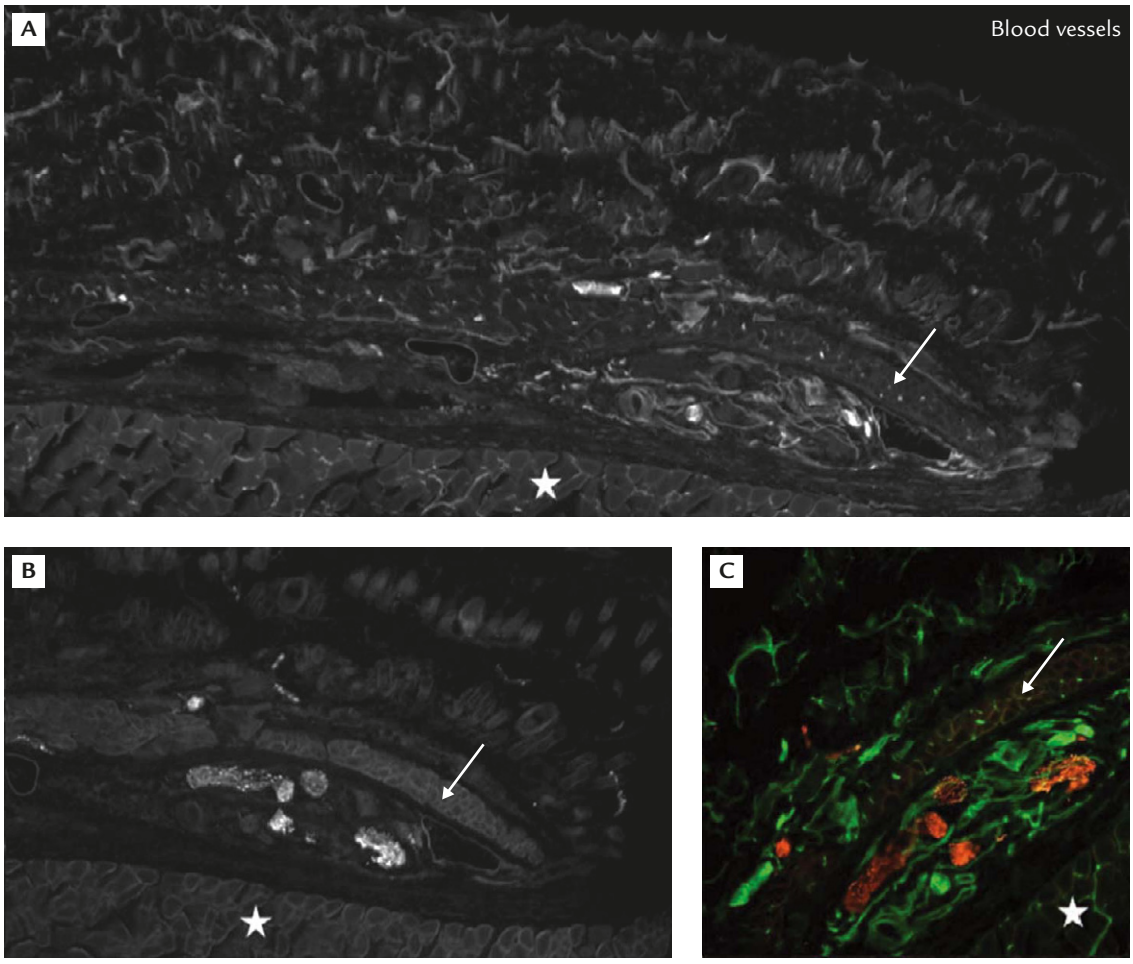

Figure 10 A transversal section image of a neurovascular section in the ST-meridian. (A) blood vessels (B) nerves (C) merged. It clearly revealed the position of the plexus under the skin skeletal muscle (arrow) and above the abdominal muscle layer (☆).

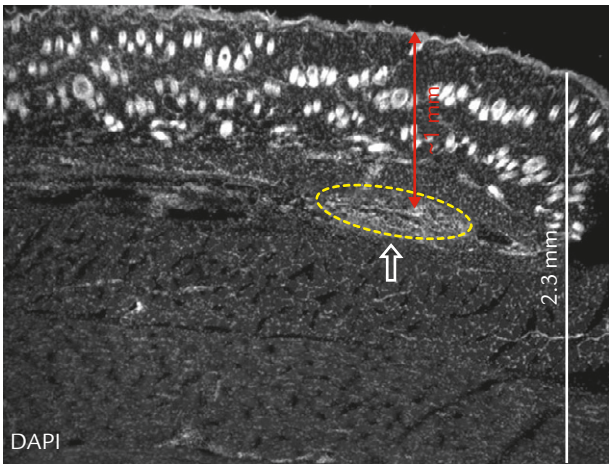

Figure 11 The image showing nuclei distribution revealed by DAPI staining in the same tissue as shown in Figure 10. The marked region (open arrow) is the neurovascular plexus.

The BHC is surrounded by smooth muscle-like muscles [1] but we could not detect such muscles around the 'head' part of the neurovascular plexus. More importantly, the coil-like blood vessel is supposed to form a bundle with the BHD, but we could not identify such ducts in our samples. Thus, it remains an unsolved problem whether the plexus of coiled blood vessel is the anatomical structure corresponding to an acupoint.

Nevertheless, there is some supporting evidence for identifying the plexus described in Figures 7 to 10 as BHC: It was found that there were chromaffine cells in the plexus [25,26], where these cells were characteristic cells in BHC [1]. The presence of the chromaffine cells which produces adrenalin/noradrenalin hormone is a hall mark of BHCs, and may have significant physiological functions that accounts acupuncture therapy. Another positive piece of evidence is the flowing out of chrome-hematoxylin through the perforation when it was injected into the muscle corresponding to the K-line (Figure 6B). It suggests that the plexus is somehow connected to the acupuncture muscle channel [27]. Thus, further studies are in need to elucidate the identification of the neurovascular plexuses with acupoints or BHCs.

Acknowledgments

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