경락추적을 위한 피내 알시안 블루 주입방법

목적 : 본 논문에서는 경락을 추적하기 위한 피내(皮內) 알시안 블루(Alcian blue) 염색 방법을 기술한다.

방법 : 1% 알시안 블루 용액을 31가지 바늘이 달린 0.5mL 인슐린 주사기를 사용하여 경혈 지점에 피내 주사한 후 실제 현미경 하에서 수술, 관찰하였다. 면역조직화학적 방법을 사용하여 해당 조직을 레이저 공초점주사현미경으로 관찰하였다.

결과 : 알시안 블루로 염색되고 피하로 이어지는 실 모양 구조물 관찰하였다. 이 조직 내에서 특징적인 막대모양 핵 배열 및 1-2μm 크기의 DNA 구조물 관찰되었다. 또한 경혈 조직 내에서 풍부한 모세혈관 및 백혈관 구조물, 그리고 약 300μm 크기의 소체형 구조물 확인하였다.

결론 : 경혈 지점의 피내에서 발견된 특징적 실 모양 구조물 및 소체형 구조물은 각각 표층 봉한관 및 소체로 판단된다.

Intradermal Alcian-Blue Injection Method to Trace Acupuncture Meridians

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ABSTRACT

Objective In this article, we report on the intradermal Alcian blue staining method for tracing the meridians of acupuncture.

Methods 1% Alcian blue solution was injected into acupoints by using a 0.5mL insulin syringe with a 31-gauge needle, then the skin was incised and was observed under a stereoscopic microscope. The specimens were examined by using immunohistochemical methods and were observed under a confocal laser scanning microscope.

Results A threadlike structure, which was visualized with Alcian blue, existed in dermis layer and proceeded to hypodermis. In this structure, characteristic alignments of rod-shaped nuclei and 1-2μm sized DNA granules were observed. Furthermore, abundant blood capillary plexuses, peripheral nerve endings, and a corpuscle-like structure (about 300μm in diameter) were visualized in the skin tissues of acupoints.

Conclusion It was concluded that the specific threadlike and corpuscle-like structures corresponded to superficial Bonghan duct and corpuscle, respectively.

Key words Acupoint, Intradermal injection, Alcian blue, Tracing, Superficial Bonghan duct and corpuscle, Immunohistochemistry, Drug delivery

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1. Introduction

Traditional oriental medicine involving acupuncture and moxibustion is based on the theory of meridians and collaterals(12 meridians and 8 extra-meridians). The meridians and collaterals have been considered as the specific channels for the circulation of qi. The twelve regular meridians are composed of three yang and three yin meridians, of the hands and the feet, respectively. The channels penetrate five zang, six fu, and meridians of the hands and the feet, respectively.

There have been several efforts to visualize the classical meridians of acupuncture by injecting specific agents, especially radioactive tracers, into the skin. Among them the paper of Bonghan Kim was the first report on the meridian tracing: In 1963, he conducted an experiment for observing the circulation paths of meridians by injecting PI2 into acupoints of rabbit. Three hours after the injection, the radioactive dosimetry of the tissues corresponding to the classical meridians appeared far higher than that of surrounding tissues.

It is notable that only Kim was able to find a specific anatomical structure in the classical acupoints, which was called "superficial Bonghan(BH) corpuscle". According to his results, a BH corpuscle was connected to 2-3 BH ducts, which proceeded along the direction of meridians, or to the deep viscera. The superficial BH corpuscles and ducts were parts of a circulatory system which was consisted of several subsystems: superficial BH system in the skin, intravascular BH system inside large blood and lymphatic vessels, and organ-surface BH system on the surfaces of various internal organs. The intravascular BH ducts inside blood vessels were confirmed by using an acridine-orange staining method, and those inside lymphatic vessels were observed employing a Janus Green B staining technique as well as a nanoparticle injection method.

Recently a series of investigations have been performed in order to elucidate the characteristics, looking into: (1) the anatomy and the basic histology using conventional staining methods and confocal laser scanning microscopy (2) the ultrastructure by using various electron microscopy such as scanning electron microscopy(SEM), transmission electron microscopy(TEM), cryo-SEM, and SEM with a focused-ion-beams(FIB/SEM) and (3) the cellular nature by using immunohistochemistry, and (4) the circulatory function by injecting Alcian blue or nanoparticles. The present work is mainly concerned with the first and the last categories, and partly with the third category.

In this article, we report on the intradermal Alcian blue staining method for tracing the meridians of acupuncture. A threadlike structure was visualized by injecting Alcian blue solution into classical acupoints. It was concluded that the threadlike structure was the superficial BH duct on the basis of characteristic alignments of rod-shaped nuclei and the existence of 1-2 μm sized DNA granules in the tissue. Furthermore, in the acupoints, a corpuscle-like structure with abundant blood capillary plexuses and peripheral nerve endings was visualized by using immunohistochemical staining methods. This structure was interpreted as the superficial BH corpuscle.

II. Materials and Methods

1. Animal preparation

Male Wistar rats of 10-12 weeks old were used in this study. The animals were housed in a temperature-controlled environment(23°C) with 60% relative humidity with a 12-h light/dark cycle. The animals had ad-libitum access to food and water. The procedures involving the animals and their care conformed to institutional guidelines, which were in full compliance with current international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996).

2. Surgical procedures

The animals were anesthetized with urethane(1.5 g/kg) administered intraperitoneally. All experimental procedures including surgery were performed under deep anesthesia. The animals were kept in the sternal recumbency position. Then the dorsal part of the body was shaved with a clipper. Thirty minutes after anesthesia 0.3 mL of the filtered 1% Alcian blue solution(2.0 mg/mL, pH 7.4) was administered slowly into ST36 in stomach meridian and lumbar-sacral point(LSP) in governing vessel(GV), respectively, by using a 0.5 mL insulin syringe with a 31-gauge needle(BD Ultra-Fine II, Becton, Dickinson and Company, NJ, USA). Two hours after injection, the dorsal skin was incised, from the LSP region to the scapula region, with operation knives and scissors. The stained parts of the skin were observed under a stereoscopic microscope(SXZ 12, Olympus, Japan), and images were taken by a digital camera(Nikon, Japan) and a CCD camera(DP 70, Olympus, Japan). After observation, the Ab-stained regions were sampled for histological analyses.

3. DAPI staining

The samples were fixed in 4% paraformaldehyde (PFA) solution for 1 hour, washed with phosphate buffered solution (PBS), and then stained with DAPI solution(SlowFade Gold, Invitrogen, Oregon, USA) for 10 minutes in a dark place. After staining, the samples were covered by a coverslip and examined under a phase contrast microscope (BX51, Olympus, Japan) at the excitation wavelength of 350 nm for detection of DAPI fluorescence (emission wavelength: 470 nm) from cell nuclei.

4. Immunohistochemistry

The skin biopsies(5mm) were fixed in a fixative consisting of 0.5% paraformaldehyde(Sigma, USA) and 15%(v/v) saturated picric acid(Sigma, USA) in 0.1M sodium phosphate buffer(SPB, pH7.0) for 2 hr at room temperature, followed by several times of wash in SPB(pH7.4)(Hashimoto et al., Methods Enzymol. 1999;307:84–107). Prior to sectioning, the tissues were infiltrated in an OCT compound(Sakura Finetek, USA) and frozen in isopentane(Fluka, Germany) at the temperature of liquid nitrogen. The frozen specimens were placed on dry ice or in the -20°C cryostat and then sectioned by using a cryotome(Microm Lab. HM 505E, Germany) at 100 μm. Frozen tissue sections of skin were then rinsed in PBS for 15 min to remove OCT embedding material and incubated in a 3% sodium deoxycholate solution(Fluka, Italy) for 4 hr at room temperature under mild agitation in a 24-well plate(Falcon, USA). The specimens were rinsed twice with distilled water and then with PBS for three times 1 hr each. After this, the sections were incubated in 10% normal goat serum overnight at 4°C to minimize non-specific binding. To stain blood vessels, sections were incubated with mouse monoclonal RECA-1 antibody(diluted 1:50, 1 day, Abcam, UK). Visualization was performed by using Alexa Fluor 488-conjugated goat anti-mouse IgG(H+L)+diluted 1:500, 1 day, Invitrogen, USA). All incubations of thick sections with primary and secondary antibodies were carried out at 4°C. Washes with PBS were performed between each step, and all antibodies were diluted in PBS containing 10% normal goat serum and 0.01% NaN3. After a final wash, the sections were stained with propidium iodide(PI) and mounted in an antifading medium(Invitrogen, USA). The slides
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There have been several efforts to visualize the classical meridians of acupuncture by injecting specific agents, especially radioactive tracers, into the skin\(^\text{3-5}\). Among them the paper of Bonghan Kim was the first report on the meridian tracing: In 1963, he conducted an experiment for observing the intravascular BH ducts inside the deep viscera. The superficial BH corpuscles and acupoints, which was called "superficial Bonghan(BH) specific anatomical structure in the classical meridians" was visualized by using immunohistochemical staining methods. This structure was interpreted as the superficial BH corpuscle. According to his results, a BH corpuscle-like structure with abundant blood capillary plexuses and peripheral nerve endings was visualized by using immunohistochemical staining methods. This structure was interpreted as the superficial BH corpuscle.

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nuclei were 10-20 μm in diameter. The nuclei were rod-shaped and aligned along the Alcian-blue-stained threadlike structures. The morphology of these nuclei was characterized by the plexus of blood capillaries. Immunohistochemical data revealed the specific distribution of BH ducts and corpuscles large amount of and many kinds of cells were found.

By the method of DAPI staining, it was revealed that the Alcian-blue-stained threadlike structures had characteristic rod-shaped nuclei(Fig. 2). The rod-shaped nuclei were aligned along the Alcian-blue-stained part. The length and diameter of the nuclei were 10-20 μm and 2-5 μm, respectively. The separation of two neighboring nuclei varied in 20-50 μm. In the Alcian-blue stained region, the DAPI-stained cells in threadlike structures were relatively regularly aligned in one direction, whereas in the surrounding tissue the cell distribution was random. Especially, it was rarely found that DNA granules of 1-2 μm in diameter existed in the Alcian-blue-stained area, although it could not be possible to observe such granules in the other parts.

In the acupuncture points we could observe well-developed plexuses of blood capillaries(Fig. 3). A classical acupoint was located in a capillary plexus, which size was about 1.2 mm. The plexus, which was made during the injection, was also observed: Its diameter was 250 μm. It was found that the Alcian blue solution was diffused from the injection point to the surrounding tissues. Several Alcian-blue-stained spots(diameter: 10-20 μm) scattered around the injection point. Immunohistochemistry revealed that there exist abundant blood capillaries(diameter: 20-50 μm) and peripheral nerve endings(diameter: 100-200 μm) in the acupuncture points(Fig. 4).

In addition, specific corpuscle-like structures(autolysosomes, CV12, and CV14) were detected in dermal and hypodermal layer of rat skin specimens(Fig. 5). This figure was meaningful that the corpuscles were characterized by the plexus of blood capillaries. Immunohistochemical data revealed the specific corpuscle-like structures in dermal and hypodermal layers of rat skin. The corpuscle-like structure was measured about 300 μm in diameter and had elliptic shape which was specified by characteristic distribution and high density of cells. Furthermore, the corpuscle was penetrated and surrounded by fine blood capillaries.

However, these methods such as CLSM or immunohistochemical staining are limited to reveal the detail structures of basement membrane or outlayer in Bonghan corpuscle.

IV. Discussion

In 1963, it was reported that the superficial BH corpuscles were located in the reticular layer of the skin on the acupuncture meridians(3,17). A BH corpuscle had an oval shape with a long diameter of 1.0-3.0 mm and a short diameter of 0.5-1.0 mm. In addition, the superficial BH duct, which was linked to the superficial BH corpuscle, had a threadlike structure surrounded by connective tissues, and contained distributed blood capillaries. The BH duct had unique rod-shaped endothelial nuclei. Inside the duct, there existed specific liquid(BH liquid), which contained hyaluronic acid, neurohormones, and DNA microgranules. It can therefore be concluded that the Alcian-blue-stained threadlike structure and the corpuscle-like structure correspond to the superficial BH duct and corpuscle, respectively, according to the following bases:

(a) When the structure was cut, any hemorrhage was not observed. No erythrocytes were found from the structure in the field of the microscope. Morphology and physical dimensions of the threadlike structures agree with those of BH ducts and corpuscles in skin, blood vessels, lymphatic vessels, and on the surfaces of internal organs(Figs. 1, 2, 5). In all those cases, the diameters of BH ducts and corpuscles measured in the range of 20-300 μm and 100-1000 μm, respectively. Furthermore, the morphology of BH corpuscles showed oval or elliptic shape, and especially in superficial corpuscles, the base of each corpuscle was connected to a highly developed capillary system. Inside the corpuscles large amount of and many kinds of cells were found.

(b) The aligned distribution of rod-shaped nuclei, which was shown in Fig. 2, is the primary histological criteria to discern BH ducts from other tissues, such as blood and lymphatic vessels. In the case of nerves, the similar type of nuclear distribution is observed, but the dimension of nucleus and the separation of neighboring nuclei are quite different from those of BH ducts: the longer nuclear length(20-50 μm) and the shorter separation of nuclei(10-30 μm), as shown in Fig. 4.

(c) Strong stainability with Alcian blue is known to be a general characteristic of BH ducts and corpuscles(Fig. 11): it was reported that lymphatic and organ-surface thread like structures(BH ducts and corpuscles) have stronger affinity and stainability to Alcian blue compared with the other tissues. This means there exist abundant mucopolysaccharides(hyaluronans) in the threadlike structures, since the Alcian blue is known to form reversible electrostatic bonds between the cationic dye and the anionic sites on the polysaccharides.9

(d) The existence of DNA granules of 1-2 μm in diameter supports the possibility of the threadlike structures being BH ducts(Fig. 2). This characteristic corresponds with the Feulgen reaction result of organ-surface BH ducts(11). Recently this DNA granule inside the organ-surface BH corpuscle was imaged by using transmission electron microscopy(10) and atomic force microscopy(12). It was supposed that the DNA granule, named as BH microcell, acts like a multipotent stem cell(13).

It is important that here we confirmed the abundant existence of blood capillary plexuses and peripheral nerve endings in acupuncture points, which corresponds to previous reports(14,15).

The micro-spots stained by Alcian blue inacupoints (Fig. 3) can be regarded as the dermal mast cells in the basis of the paper of Zhu and Xu, in which it was reported that there concentrated more mast cells in acupuncture meridians than in the surrounding tissues(16).

In summary, we found an evidence for specific anatomical structures in acupoints by using an intradermal Alcian blue injection method. In addition, by using histological and immunohistochemical examinations it was concluded that the structures correspond to the superficial BH corpuscles and ducts, which were first found by Bonghan Kim in 1963. Since it was reported that the superficial BH ducts could transport mucopolysaccharide ride-contained liquid form acupoints to the inner side of body or through the classical meridians, it will be possible to explain the mechanism of herbal acupuncture(or pharmacoacupuncture) in an anatomical basis(17). Furthermore, one may be able to apply this method to the drug delivery: it will be possible to deliver a specific drug to a target organ by injecting the drug into a classical acupoint(18). However, finally we should indicate that this study is just an evidence for the anatomical entity of acupuncture points and meridians, but not a conclusive proof.

More precise and structural studies and improvements of the experimental method are needed.

V. Acknowledgement

This work was supported by KOSEF/NRL M1-
were observed with a confocal laser scanning microscope (Zeiss LSM 510, Germany).

III. Results

We could find Alcian-blue-stained threadlike structures, which proceeded from acupuncture points to hypodermis (Fig. 1). The length and diameter of the threadlike structures were 2-3cm and 200-400μm, respectively. In the injection point, the Alcian blue solution diffused into the surrounding tissues in the range of 2-3mm. We could also observe the Alcian-blue-contained small lymph vessels (diameter: 10-30μm) around the injection points, although there were no such vessels around the threadlike structures.

By the method of DAPI staining, it was revealed that the Alcian-blue-stained threadlike structures had characteristic rod-shaped nuclei (Fig. 2). The rod-shaped nuclei were aligned along the Alcian-blue-stained part. The length and diameter of the nuclei were 10-20μm and 2-5μm, respectively. The separation of two neighboring nuclei varied in 2-3mm. We could also observe the Alcian-blue-containing small lymph vessels in threadlike structures were relatively regularly aligned in one direction, whereas in the surrounding tissue the cell distribution was random. Especially, it was rarely found that DNA granules of 1-2μm in diameter existed in the Alcian-blue-stained area, although it could not be possible to observe such granules in the other parts.

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V. Acknowledgement

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VI. References


establishment grant” provided by Gwangju Institute of Science and Technology in 2008.

VI. References


Figure Legends

Fig. 1. The stereo-microscopic image of a threadlike structure stained by Alcian blue (arrowhead).

Fig. 2. Merged image of the phase contrast and the DAPI fluororescent images of the threadlike structure stained by Alcian blue at 100 point. Rod-shaped nuclei (arrowheads) are aligned along the Alcian-blue-stained part (broken lines). We can see that a DNA granule of 1–2 μ in diameter (arrow) exists in...
Fig. 3. Bright field image of thick section (150 μm) of rat skin (location: CV12, depth: 1050 μm) after Alcian blue injection. In the acupuncture points one can observe well-developed plexuses of blood capillaries (triangle of dashed lines). The puncture (P), which was made during injection, can be also observed (diameter: 200 μm). Alcian blue solution is diffused from the injection point to the surrounding tissues.

Fig. 4. Immunohistochemical image of the skin sample of Fig 3. Staining was done by DAPI for nuclei (blue), anti-NF-M antibody for peripheral nerves (red), and RECA-1 antibody for blood vessels (green). One can observe a peripheral nerve ending (SN) and a hair follicle (HF), and blood capillaries (C). Scale bar represents 100 μm.

Fig. 5. A CLSM image of the immunohistochemical result showing a specific corpuscle-like structure in the dermal/hypodermal layer of rat skin (CV12). The corpuscle-like structure had elliptic shape (broken line) which was revealed by characteristic distribution and high density of cells (P1).