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RESEARCH ARTICLE

Acupuncture Muscle Channel in the Subcutaneous Layer of Rat Skin

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Abstract
Using a mixed-dye injection technique, we found a novel kind of muscle fiber with a lumen, established its precise location in the subcutaneous muscle layer along the acupuncture muscle of the bladder line, and determined its detailed ultrastructure. The channels with flowing liquid were a novel kind of muscle fibers with lumens and they were located in the subcutaneous muscle layer of rat. Their detection was realized by using chrome-hematoxylin and a mixture of fluorescent nanoparticles and commercial Pelikan ink. These acupuncture muscle channels were hidden among the neighboring skin skeletal muscle fibers and were barely distinguishable from them with light microscopes. Only with a transmission electron microscope were their characteristic features shown to be different from normal skin skeletal muscle. These features included undifferentiated muscle fibers that resembled immature myofibrils without Z-lines and reassembled telophase nuclei.

1. Introduction

Acupuncture is one of the most widely accepted alternatives to Western medicine, yet its mechanism of action is not scientifically understood. A critical question is whether the acupuncture meridian is a real physical being with channels containing flowing liquid or merely an imaginary curve in the skin with some unknown nerve connections. Some evidence that support the real physical existence of acupoints and meridians are the flow of radioisotopes along meridians [1,2], a low electrical impedance at acupoints [3,4], thermal and sense propagation [5] and a low fluid resistance in the conception vessel of a pig [6]. Until now, however, no anatomical or histological structures corresponding to the acupuncture meridian have been found [7–11]. The only claim ever made in this regard was by Bonghan Kim, who discovered anatomically distinctive, threadlike ducts at the meridians of human and animal skin [1]. His claim was not confirmed by others [12] and was criticized by Kellner [13].

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Only very recently have Bonghan ducts (BHDs) been rediscovered inside blood vessels [14], on the surfaces of internal organs [15] and inside lymphatic vessels [16,17]. The speed of flow in BHDs on organ surfaces was measured to be 0.3 mm/sec, quite slow compared with lymphatic flow, let alone blood flow [18]. Neurotransmitter hormones, adrenaline and noradrenaline were found to be biochemical components in the liquid [19], as was described by Bonghan Kim [20]. In this series of work, many of Bonghan Kim's claims have been confirmed, but acupuncture meridians, that is, BHDs in skin, were not disclosed due to the complexity of skin and lack of techniques to discern the BHD from the surrounding tissues. An elementary investigation uncovered a threadlike structure at the lumbar sacro point and ST36 by using the staining dye Alcian blue [21]. However, no histological study has shown striated muscle fibers with a lumen structure.

The current work reports our unexpected discovery in the course of searching for the acupuncture meridian. In the classic canon of Traditional Chinese Medicine, Yellow Emperor's Naijing Volume II, there is an attempted morphological description on acupuncture point systems in three chapters: acupuncture meridian (chapter 10), acupuncture collateral (chapter 11) and acupuncture muscle (chapter 13). The acupuncture muscle is called such terms as muscle meridian, muscle group, or meridian muscle group, and its anatomical structure is not known except for a vague notion as a muscle group corresponding to each meridian. Bonghan Kim claimed to find anatomical structures corresponding to the acupuncture meridians and collaterals, but did not mention the acupuncture muscle at all [20]. We observed, for the first time, an anatomical structure corresponding to the acupuncture muscle which was different from BHDs.

In the present work, nanotechnology was applied to reveal the hitherto unobserved novel muscle fiber with a lumen. This channel of the lumen was found at the acupuncture muscle of the bladder meridian line (BL) of a rat; hence we named it the acupuncture muscle channel (acuchannel). It was noticed by injecting a mixed dye of chrome-hematoxylin and DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate) and another mixed dye of commercial Pelikan ink and fluorescent nanoparticles in the subcutaneous layer of the dorsal skin. The acuchannel was *in situ* visualized by the black color of the flowing dye.

Samples of the tissue were examined under a phase contrast microscope, and fluorescence from the nanoparticles was essential to identify the acuchannel. Without the nanoparticles, the muscle fiber that contained the lumen, that is, the acuchannel, would have been mistaken for one of the skeletal muscle fiber in the subcutaneous layer of the skin because the lumen was collapsed and hardly noticeable. Further detailed study of the acuchannel by using a transmission electron microscope (TEM) showed that the acuchannel had characteristic features of growing undifferentiated muscle fibers.

2. Methods

2.1. Animal preparation and surgical procedure

Rats (Wistar, both sexes, around 200 g) were obtained from Jung-Ang Laboratory Animal Company for this study. The animals were housed in a constant, temperature controlled environment (23°C) with 60% relative humidity under a 12 hour light/dark cycle. All rats had *ad libitum* access to food and water. The procedure 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 rats were anesthetized with urethane (1.5 g/kg) administered intraperitoneally allowing, all surgical procedures to be performed under systemic anesthesia.

2.2. Injection dye

In order to visualize an acuchannel in rat skin we used two different mixtures of staining dyes. (1) Chrome-hematoxylin and DiI, (2) Fluorescent nanoparticles and Pelikan ink (Pelikan 4001, Germany). Chrome-hematoxylin was mixed with DiI (1:1 by volume). Chrome-hematoxylin is useful for visualization under light stereomicroscopy and DiI is used for proving the existence of phospholipids, a main component of cell or plasma membranes. In order to examine the ultrastructure of the acuchannel by TEM, we used fluorescent nanoparticles (FNP). The FNPs were synthesized employing a modified polyvinylpyrrolidone method and cobalt ferrite magnetic nanoparticles coated with a shell of amorphous silica, which contained a derivatized Rodamine B fluorescent dye on the inside of the silica shell and biocompatible polyethylene glycol on the outside [22]. They were mixed with commercial Pelikan ink (1:1 by volume; Pelikan 4001, Germany) and diluted into 1.0 mg/mL of FNP by using phosphate buffered saline (pH=7.0). The Pelikan ink was used for *in situ* visualization under light stereomicroscopy and the FNPs were used for dual purposes: Firstly, the fluorescence from the nanoparticles was used to identify the acuchannel

from among the neighboring skeletal muscle fiber, which look indistinguishably similar to the acuchannel under light microscopy without the fluorescence of the FNP. Secondly, TEM examination was able to demonstrate FNPs flowing in the lumen of the acuchannel.

2.3. Visualization method and microscopy

Under deep systemic anesthesia, the longitudinal midline of the dorsal skin of rats and the longitudinal lines corresponding to bladder lines (BL; dashed lines in Figure 1) were marked for reference. The longitudinal midline and the transversal line at the lumbar sacro point of the dorsal skin of rat were incised and the skin was turned over to expose the subcutaneous side of the skin. We injected a mixed dye of chrome-hematoxylin and DiI into the subcutaneous muscle layer of the rat skin. Immediately after visualization by using chrome-hematoxylin and DiI, we isolated the specimen of skin with the visualized acuchannels. This specimen was examined with a phase contrast microscope in bright field and fluorescent modes (Figure 2).

For TEM examination, samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate

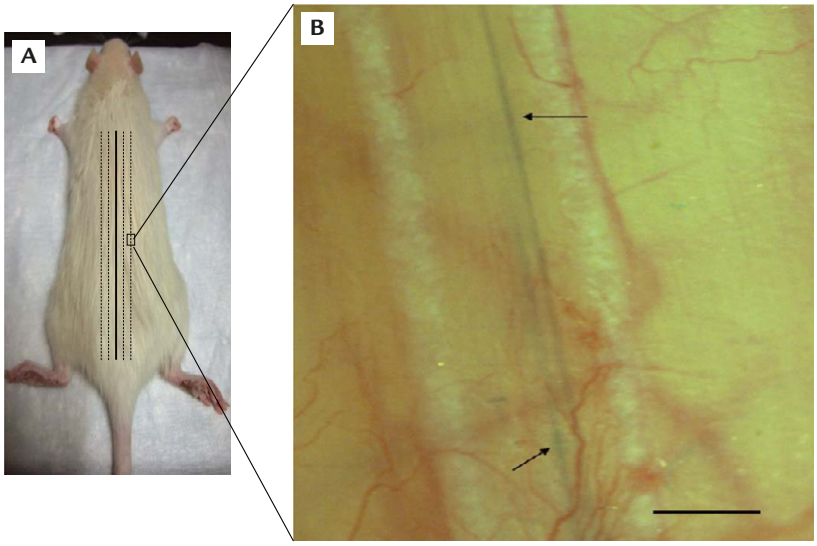

Figure 1 (A) Dorsal skin of a rat. The midline and the transverse line near the lumber sacro point were incised, and the right hand skin was turned over to expose the subcutaneous side into which the staining dye was injected *in situ*. The dotted lines correspond to the left and the right bladder meridians. Each meridian has two lines. (B) Magnified view of the subcutaneous side corresponding to the boxed area in (A). The two straight lines (arrows) are the acuchannels that were visualized by the flow of chrome-hematoxylin and DiI (1:1 by volume) into the skeletal muscles in the hypodermis. Scale bar, 0.5 mm.

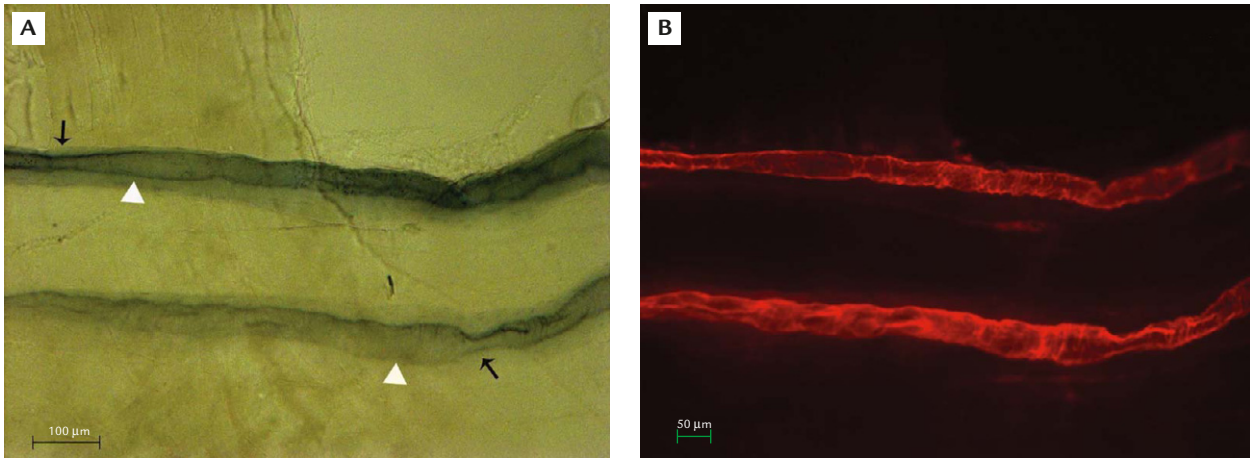

Figure 2 Light microscopic image (A) showing the flow of chrome-hematoxylin and fluorescence light microscopic image (B) displaying the fluorescence from DiI in the acuchannels taken from the visualized straight lines by injection of chrome-hematoxylin and DiI [1:1 (v/v)]. There were two parallel channels in the acupuncture muscle group corresponding to the bladder line. The channels were not uniform in thickness (thick part, Δ; thin part, arrow).

buffer at 4°C for 4 hours. The specimens were post-fixed in 1% OsO_4 in 0.2 M sodium cacodylate buffer for 1 hour; stained in 0.5% uranyl acetate; dehydrated with ethanol and propylene oxide and embedded in epoxy resin (Epon 812). Ultrathin sections were collected on large-scale copper grids, contrasted by 2% uranyl acetate and Reynolds' lead citrate, and then examined with a TEM (JEM1010, JEOL, Japan).

3. Results

An acuchannel was found at the muscle group of the BL of a rat (Figure 1). The staining dye, chrome-hematoxylin, nearly flowed in a straight line through this channel. It was manifestly not a blood vessel because its color was not red. Its straightness was an apparent feature that distinguished it *in situ* from lymphatic vessels. Its features, which were distinctly different from those of blood or lymph vessels, were convincingly disclosed in the TEM images.

As shown in Figure 2 there were two parallel acuchannels whose lumens were stained by both chrome-hematoxylin (A) and DiI (B). Their thicknesses were about 50 μm. The fluorescent image of DiI implied that there was phospholipid at the inner sides of the lumens, which suggested that membranous material lined the lumens.

One surprising result was the anatomical position of the acuchannel. It was located in the subcutaneous muscle layer, as marked by the arrowheads and the arrows in Figure 3. Another unexpected result was that the acuchannel was a novel kind of muscle fiber, which was barely distinguishable from other skeletal muscle fiber in its neighborhood.

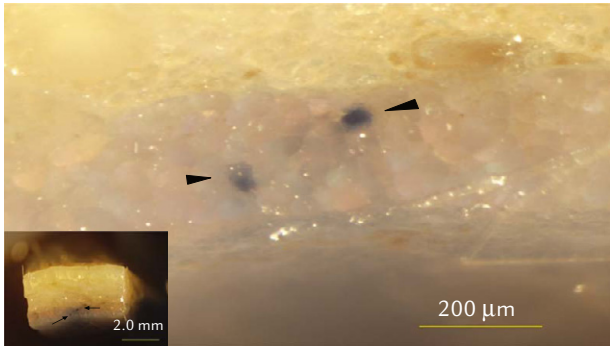

Figure 3 Stereoscopic images of two deep blue colored acuchannels (arrowheads) after injection of Pelikan ink and fluorescent nanoparticles [1:1 (v/v)]. The inset shows a specimen of whole skin around the bladder meridian line. The hair and the epidermis are on the upper side. The anatomical positions of the two acuchannels in the skin are indicated by two arrows and are in the skin skeletal muscle layer just below the hypodermis.

In a sense, the acuchannel was hidden among a crowd of similar looking skeletal muscles. Thus, its existence was not noticed in conventional histology using hematoxylin and eosin staining. Its unique distinctive feature was the existence of a lumen, yet the lumen had collapsed during the sample preparation processes and was barely detectable with a light microscope. In the current study the collapsed lumen was made visible by the bright red fluorescence from magnetic nanoparticles flowing in the acuchannel (Figure 4).

In order to investigate the detailed nature of the acuchannel, we used TEM, in which the nanoparticles were essential to locate the acuchannel among the many skeletal muscle fibers because the other indicator, Pelikan ink, was not retained during the sample preparation processes for TEM.

Electron microscopy revealed that rat acuchannels had unique structural features which were distinctively different from neighboring skeletal muscle fibers as depicted in Figure 5A. Firstly, rat acuchannels contains a lumen which was detected by fluorescent nanoparticles. As shown in Figure 5B the lumen was in a compressed state and its boundaries were not lined by a cell membrane. The lumen was located among myofibrils which displayed alternating A-bands and I-bands. However, myofibrils in rat acuchannels did not exhibit distinct Z-lines, a typical structure in striated skeletal and cardiac muscle cells. The myofibrils without Z-lines were mainly arranged around the lumen. Secondly, the main part of rat acuchannels, which had a lack of myofibrils or sarcomeres, was composed of thin

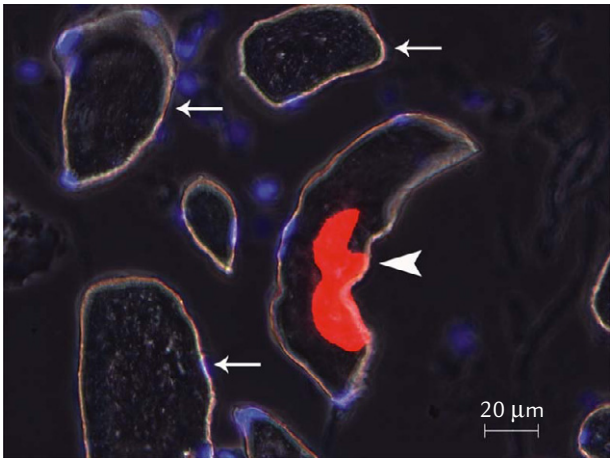

Figure 4 A merged phase contrast and fluorescence light microscope image of a cross-sectioned acuchannel, which was visualized by injection fluorescent nanoparticles (v/v, 1:1). The bright red color is due to the fluorescence of magnetic nanoparticles inside the lumen of an acuchannel. The acuchannel (arrowhead) was a novel kind of muscle fiber and was located among other similar looking muscle fibers (arrows).

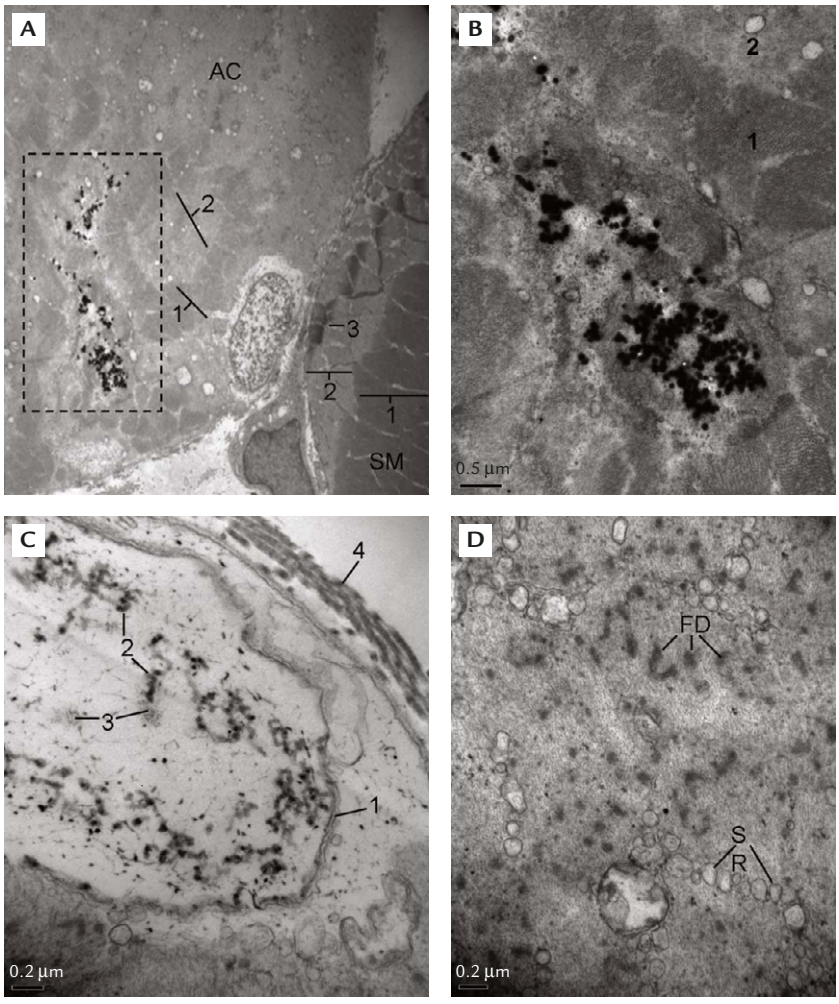

Figure 5 Transmission electron microscope images of a rat acuchannel. (A) Low-magnification view of a transverse section of a rat acuchannel (AC) and skeletal muscle fiber (SM). The rectangular area shows the lumen with flowing nanoparticles. (1) — A-band (dark band), (2) — I-band (light band), (3) — Z-line. Magnification × 4000. (B) Magnified image of the rectangular part in Figure 5A showing the distribution of scattered nanoparticles (black dots). The lumen was collapsed. A- and I-bands are denoted by 1 and 2, respectively. Magnification × 25,000. (C) Electron micrograph illustrating a nucleus of rat acuchannel at final stage of mitosis, telophase. At this stage the nucleus was composed of a newly assembled nuclear envelope (1) and decondensing chromosomes (2) with uncoiling chromatin (3). Rat acuchannel is surrounded by a layer of collagen fibers (4). Magnification × 50,000. (D) Highly magnified image of the rat acuchannel showing the I-band with numerous fusiform densities (FD) surrounded by thin filaments. The cytoplasm of acuchannel contains a well developed sacroplasmic reticulum (SR). Magnification × 50,000.

filaments and numerous fusiform densities (Figure 5D). Fusiform densities appeared as dense structures with round and oval shape which were distributed in a disordered way among thin filaments. The sarcoplasmic reticulum of rat acuchannels was well developed and appeared as a series of closely related tubular elements which tightly contact with the myofibrils and bundles of thin filaments (Figure 5D). Thirdly, a nucleus which was located on the periphery of rat acuchannels exhibited characteristics similar to the final stage of mitosis, telophase (Figure 5C). At this stage the nucleus was formed by a newly assembled nuclear envelope that surrounded decondensing chromosomes and partially uncoiled chromatin. Thus, our findings indicate that rat acuchannels exhibited all the features of growing, undifferentiated muscle fibers, i.e. the presence of immature myofibrils without Z-lines, numerous fusiform densities and reassembled telophase nucleus containing decondensing chromosomes.

4. Discussion

The current work not only presents the discovery of a novel muscle fiber with a lumen for liquid flow but also explains why the acuchannel has not been

observed even incidentally despite numerous related studies [7–11]. No one realized that it would be embedded among the skin skeletal muscle as a varied form of muscle fiber. Its identifiable mark under light microscopy would be its lumen, which, however, would collapse in the preparation processes for conventional histological study, therefore, could not be detected. TEM would unveil the ultrastructure of the acuchannel and allow it to be discerned from other skeletal muscle fibers, but its high magnification requires prior knowledge of the acuchannel location. Without prior information on the position of the acuchannel, it is not practically possible to apply TEM to search for the acuchannel.

One possible resolution to the problem of finding an acupuncture meridian would be to use staining dyes that flowed in the acupuncture meridian, and Bonghan Kim, indeed, mentioned that he had used a blue dye, but kept the identity of that dye secret [1,20], which was the main reason no one could reproduce his results. We have not been able to identify a BHD, but instead discovered hitherto an unknown acuchannel. Acupuncture muscles are groups of muscles running near meridians. It has been clinically used, but its anatomical structure was completely unknown. We found the surprising fact that the acupuncture muscle was a special kind of skeletal muscle with a lumen.

The significance of the current work is that we found two recipes (1) chrome-hematoxylin and (2) commercial Pelikan ink and fluorescent nanoparticles, to visualize the acuchannel *in situ* under a stereomicroscope and to trace them, even when the lumen was collapsed as shown in Figure 5, in the histological specimen thanks to the nanoparticles. With this successful strategy, we could continue the TEM study to reveal the detailed nature of the acuchannel. In that study, nanoparticles again played an essential role that is, keeping track of the acuchannel even after the washing processes during sample preparation. The nanoparticles remained in the lumen, while the dye Pelikan ink did not. Without TEM, the muscle nature of the acuchannel would not have been disclosed.

Previous works on channel flow with radioisotopes [1,2], sensation of propagation [5], and others provided no firm proof, but only weak support for the physical reality of the acupuncture meridian because of their poor spatial resolution and lack of histological data and because there can be flow in the skin through interstitial channels, lymphatic vessels or even acuchannels. If such known flow paths are to be excluded, a TEM study showing the ultrastructure is critically necessary, and so far no such thorough study has been reported.

The acuchannel in the current work may have a close relation with a BHD in the sense that it is a channel of liquid flow at the meridian muscle site. Yet, it is not a BHD. Kim described a BHD as having multiple subducts and surrounding membranes, but he also mentioned that there were BHDs with a single channel [20]. Because the two acuchannels had only single channels and because no membrane surrounded both of them, together they may be two independent BHDs. However, he described the BHD as being formed of smooth muscle-like cells, but our acuchannel was not a smooth muscle. Furthermore, the acuchannel did not have an inner membrane nor was it connected to an acupoint (Bonghan corpuscle) which were two essential features of a BHD. Thus, further work is needed to clarify the relationship between the BHD and the acuchannel.

The salient points of the current work are summarized as follows: Channels with flowing liquid were discovered at the acupuncture muscle (bladder line muscle) by finding a recipe that used a mixture of chrome-hematoxylin and fluorescent nanoparticles, and its injection technique. The precise anatomical location of the acuchannel was within the subcutaneous muscle layer in rat dorsal skin and a novel kind of muscle fiber with a lumen for liquid flow was discovered.

The current work is only an initial stage toward full revelation of the acuchannel and calls for further investigation. First of all, if the acupuncture muscle is to be identified, tracking of the acuchannel for longer distances is necessary. Secondly, an immunohistochemical study to characterize the novel muscle is needed to elucidate its nature. Analysis of the liquid flowing in the acuchannel, such as microdialysis and proteomics, is desired, and its electrophysiological properties need to be studied because electroacupuncture is a widely accepted acupuncture therapy.

Acknowledgments

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