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RESEARCH ARTICLE

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Catecholamine-storing Cells at Acupuncture Points of Rabbits

Vyacheslav Ogay, Min Su Kim, Hyo Jun Seok, Cheon Joo Choi, Kwang-Sup Soh*

Biomedical Physics Laboratory, Department of Physics and Astronomy, Seoul National University, Seoul, Korea

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Abstract
Recent studies have shown that specific sites of the skin related to the acupoints contain a high concentration of catecholamines, especially noradrenaline (NA). Considering this newly discovered property of the acupoints we assumed that heterogeneous distribution of cutaneous catecholamines could be associated with a specific location of catecholamine-storing cells in acupoint sites. In the present work we used an immunohistochemical method and confocal laser scanning microscopy to examine the presence of catecholamine-storing cells at acupoints of rabbits. Double immunofluorescence staining with antibodies against adrenaline and NA revealed only the cells storing NA in the dermal layer of rabbit skin. NA-storing cells were randomly scattered as single cells as well as existing in small clusters in a globular tissue formation surrounded by blood vessels and capillaries. Microscopic analysis of histological sections also revealed that the distribution of NA-storing cells was closely associated with the location of acupoints. Thus results from our study strongly suggest that acupoint areas of rabbit skin contain catecholamine-storing cells which can release a high level of NA during acupuncture stimulation.

1. Introduction

Acupuncture, an alternative medicine method, has been widely used to treat a variety of diseases and symptoms [1,2]. Acupuncture points are discrete anatomic sites with unique functional properties in human and mammalian skin and are the focus of diagnosis and treatment in traditional Chinese medicine [3]. However, the morphological and physiological characteristics of acupuncture points are still little understood, although they have long been subjects of interest in the field of modern biomedicine.

To clarify the nature of acupuncture points and the mechanism of acupuncture treatment, many researchers have searched for distinct anatomical and histological features that might differentiate acupuncture points from the surrounding tissue. Histological investigations have revealed that acupuncture points in the skin have a number of characteristic properties such as a high density of nerve endings, A- and C-fibers [4–7], a specific distribution of well-developed blood vessel networks interconnected with nerves [8,9], a gathering of leucocytes and mast cells around a blood plexus [10,11], an

*Corresponding author. Biomedical Physics Laboratory, Department of Physics and Astronomy, 25-310, Seoul National University, Seoul 151-747, Korea.
E-mail: kssoh1@gmail.com

abundance of loose connective tissue and a significant concentration of acid mucopolysaccharides [12]. Niboyet observed that an acupoint consisted of a cutaneous nerve-vessel bundle wrapped in a sheath of connective tissue [13], whereas Heine found that the basis for acupoints were perforations of the superficial fascia in which a nerve-vessel bundle was covered with a perineural sheath [14]. Langevin studied the intimate relationship between acupoints and interstitial connective tissues [15]. However, until now there has been no published evidence for any separate specific structures corresponding to the acupoints or meridians, except for that of Bonghan Kim who claimed evidence for an anatomical substrate for acupuncture points, based essentially on experiments done in rabbits [16]. According to Kim, the acupuncture point-meridian system is, histologically, composed of a system of specialized "Bonghan corpuscles", interconnected by specialized Bonghan ducts which were found in the skin and deep in the viscera. Anatomically, superficial Bonghan corpuscles had oval shapes and were located in the reticular layer of the skin loosely embedded within the connective tissues. In addition, the corpuscle was surrounded by comparatively large blood vessels and capillaries. Structurally speaking, each superficial Bonghan corpuscle was a complex tissue structure consisting of an outer and inner layer. The outer layer comprised of mainly smooth muscle-like cells surrounded by a connective tissue sheath containing capillaries. The internal part of a Bonghan corpuscle consisted of different tissue cells, elastic connective tissues and argyrophilic fibers that intermingle with a well-developed blood vessel network. In all parts of the internal layer, chromaffin cells were observed. Chromaffin cells stained by chromium salts were distributed around the blood vessels in small groups or otherwise scattered randomly.

Thus based on anatomical and histological data, Bonghan Kim concluded that he had discovered unique tissue structures that are quite different from already-known structures in the skin such as the Meissner corpuscle, the Ruffini end organ and the Pacinian corpuscle. In addition, using physiological experiments he showed that Bonghan corpuscles can be excited after stimulation by an acupuncture needle [16].

Naturally, many scientists in China, Germany, France, Austria and the United States repeated his work but no one could confirm his discovery [17–19]. The most unfortunate fact was that the histological techniques used in his experiments were never mentioned in detail, making critical assessments impossible. Thus results obtained by Bonghan Kim were not accepted and were therefore neglected for a long time.

However, having analyzed Bonghan Kim's data on the histological characteristics of Bonghan corpuscles, we concluded that one of the critical features of the corpuscle structure is the presence of chromaffin cells at acupuncture points. These cells were identified by chromium salt staining and described in more detail than the other components of Bonghan corpuscles. Chromaffin cells are neuroendocrine cells found in the medulla of the adrenal gland and in the ganglia of the sympathetic nervous system. These cells secrete the catecholamine hormones dopamine, adrenaline (A) and noradrenaline (NA), and a variety of other neuropeptides including enkephalin. Catecholamines are released as hormones by chromaffin cells in situations of stress such as psychological stress. Moreover, it was also shown that catecholamines such as NA and dopamine may be involved in the therapeutic effects of acupuncture during acupuncture stimulation [20–22].

Thus considering that specific sites of the skin related to the acupuncture points contain a high concentration of catecholamines in comparison with non-acupoints, in the present study, we examined whether rabbit acupuncture points are associated with the presence of chromaffin cells, which can serve as a main source of cutaneous catecholamines in the acupoint areas.

2. Materials and Methods

2.1. Animals

Female New Zealand white rabbits aged 8–10 weeks (1.6–1.8 kg) were obtained from the Hanlym Laboratory Animal Company (Seoul, Korea). The animals were housed in a constant temperature-controlled environment (23°C) with 60% relative humidity. All animals were exposed to a 12 hour light-dark cycle and had *ad libitum* access to food and water. Procedures involving animals and their care conformed to institutional guidelines, which were in full compliance with current laws and policies (*Guide for the Care and Use of Laboratory Animals*, National Academy Press, 1996). Before the experiments the rabbits were anesthetized with urethane solution (1.5 g/kg) and then the abdomen area was shaved.

2.2. Determination of acupoints

The locations of the Conception Vessels meridian (CV) and acupoint CV12 were determined by an acupuncture-expert using an acupoint/meridian map of animals and humans [23,24]. A CV12 point is physiologically related to digestive function in traditional Chinese medicine. This point usually strengthens and

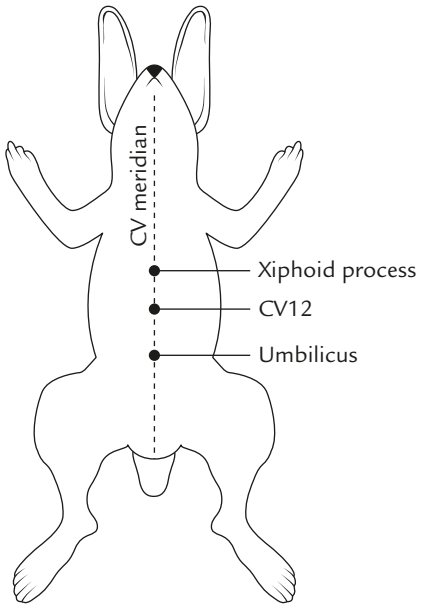

Figure 1 Schematic illustration of the location of the Conception Vessel (CV) meridian and acupoint CV12 (*Zhongwan*) in rabbits. CV12 is located on the ventral midline of the abdomen, at the midpoint between the xiphoid process and the umbilicus.

regulates the digestive organs in the body. In addition, the CV12 point can easily be identified and isolated on an animal body (Figure 1).

2.3. Immunohistochemistry

To examine the presence of catecholamine-storing cells in the acupuncture point area of CV12, we carried out a number of experiments with double immunofluorescence staining technique for thick sections, as described by Hashimoto [25]. Acupoint and non-acupoint areas (1 cm^2) of the skin with abdominal muscle walls were isolated and fixed with 0.5% paraformaldehyde in saturated picric acid overnight at 4ºC. Adrenal glands of rabbits were used as positive controls in our experiments. The specimens were washed with PBS, embedded in OCT mounting medium (Sakura Finetek, USA) and then frozen at the temperature of liquid nitrogen. The frozen specimens were sectioned in the z-plane by using a cryotome (Microm Lab HM 505E, Germany) at 100 μm. All incubations of thick sections with antibodies were carried out at 4ºC in 12-well multiplates (Costar, USA). Washes with PBS were performed between each step and all primary and secondary antibodies were diluted in PBS containing 10% normal goat serum and 0.01% NaN_3.

To enhance the penetration of antibodies the thick sections were pretreated with a 3% sodium deoxycholate solution (Fluka, Italy) for 4 hours at room temperature. The specimens were briefly rinsed with distilled water and twice with PBS for 1 hour each. After this, the sections were incubated in 10% normal goat serum overnight at 4ºC to minimize non-specific binding. In the first primary incubation, skin sections were incubated with a rabbit polyclonal antibody against NA (diluted 1:1500, 1 day, Abcam, UK), followed by a FITC-conjugated goat anti-rabbit Ig G (H+L) (diluted 1:500, 1 day, Chemicon, Temecula, CA). In the second step, the sections were incubated with a second rabbit polyclonal serum raised against adrenaline (1:1000, 1 day, Chemicon, Temecula, CA). This primary antibody was detected with Alexa Flour 555-conjugated goat anti-rabbit IgG (H+L) (diluted 1:500, 1 day, Invitrogen, Eugene, OR). To stain blood vessels, sections were incubated with mouse anti-α smooth muscle actin IgG2a (diluted 1:100, 1 day, Abcam, UK). Visualization was performed using Alexa Flour 488-conjugated goat anti-mouse IgG (H+L) (diluted 1:500, 1 day, Invitrogen, Eugene, OR). After a final wash, the sections were stained with 300 nM DAPI and mounted in an antifading medium (Invitrogen, Eugene, OR). The slides were observed with a fluorescent stereo-microscope (MVX-10, Olympus, Japan) and a multi-photon confocal laser scanning microscope (Zeiss LSM 510 NLO, Germany).

2.4. Measurement of the location of NA-positive cells in skin sections

Measurement of the location of NA-positive cells was performed according to the outlined procedure which is illustrated in Figure 2. As shown in Figure 2A, before measurement, the immunostained sections were observed by fluorescent microscope (BX51, Olympus, Japan) to detect approximate location of NA-positive cells in the slide. After that the slide was examined with a fluorescent stereo-microscope (MVX-10, Olympus, Japan) and photographed at low magnification (20×) by CCD camera (DP70, Olympus, Japan). A small rectangle in the image (Figure 2B) indicates an exact position of NA-positive cells which were detected in the fluorescence image of Figure 2A. The measurement of the distance from the point indicated by the acupuncturist to the position of NA-positive cells was performed in two-dimensional plane (x and y). Using an option "Scale" in software DP controller (Olympus, Japan) we measured a size of the section and determined the exact location of NA-positive cells. Using this procedure, the actual results were presented as a point in the graph (Figure 2C).

3. Results

The results of immunohistochemical analysis are presented in Figure 3. Staining of the skin sections of

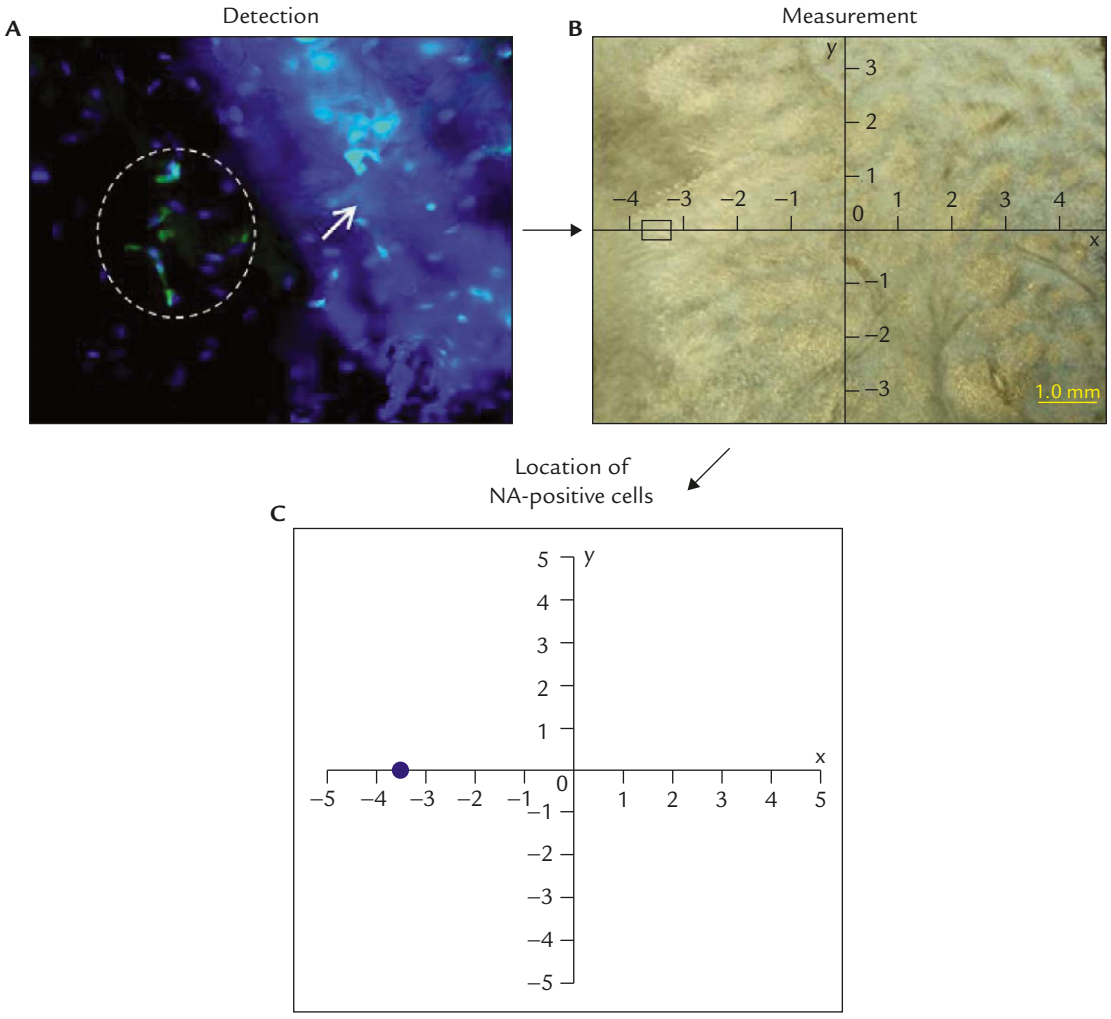

Figure 2 Measurement of the location of noradrenaline (NA)-storing cells in skin sections (CV12). (A) NA-storing cells (circle) are shown as a small cluster which was located near a blood vessel (arrow). Cell nuclei (blue) were stained with DAPI. Magnification 400×. (B) Low-magnified image shows the distance from the center point (acupoint) to the location of NA-storing cells (rectangle). (C) The data on the location of NA-storing cells are presented as a point in the graph. The distance from the centre to the location of the NA-storing cells is given in mm.

CV12 with polyclonal antibodies against NA and A remove round brackets revealed the presence of catecholamine-positive cells containing NA, whereas immunoreaction for A was negative (Figures 3A and B). These data were simultaneously compared with positive control sections of adrenal glands in which NA- and A-storing chromaffin cells were observed (Figures 3C, D, F). Furthermore, staining of skin and control sections with a secondary antibody alone, or with non-immune serum, were negative. As shown in a merge image (Figure 3C), NA-storing cells were randomly scattered as single cells and also as small clusters in a globular tissue formation at a depth of 500–600 μm from the epidermis. These cells measured about 10 μm in diameter and had a round shape, while in other samples they had an oval morphology and were bigger in size. Data on the morphological characteristics of NA-storing cells are summarized in Table 1. Our data also showed that the positions of NA-storing cells in six specimens (CV12) were different. As shown in Figure 4 and Table 2, NA-storing cells in skin sections (1 cm^2) were located about 2–4 mm away from the point indicated by the acupuncturist. Moreover, the data in Table 2 show that catecholamine-storing cells were scattered in a diameter of about 150–200 μm in the dermal layer of the skin at a depth from 300–600 μm from the epidermis.

In another series of experiments, the staining of thick sections of CV12 with α-smooth muscle actin antibody and DAPI revealed the globular tissue formation which was surrounded by blood vessels and capillaries (Figures 5B–D). Stereomicroscopic observation showed that this globular formation was located in the reticular layer of rabbit skin at a depth of 600–700 μm. The position of this structure in the skin section was a few millimeters away from the center point (Figure 5A).

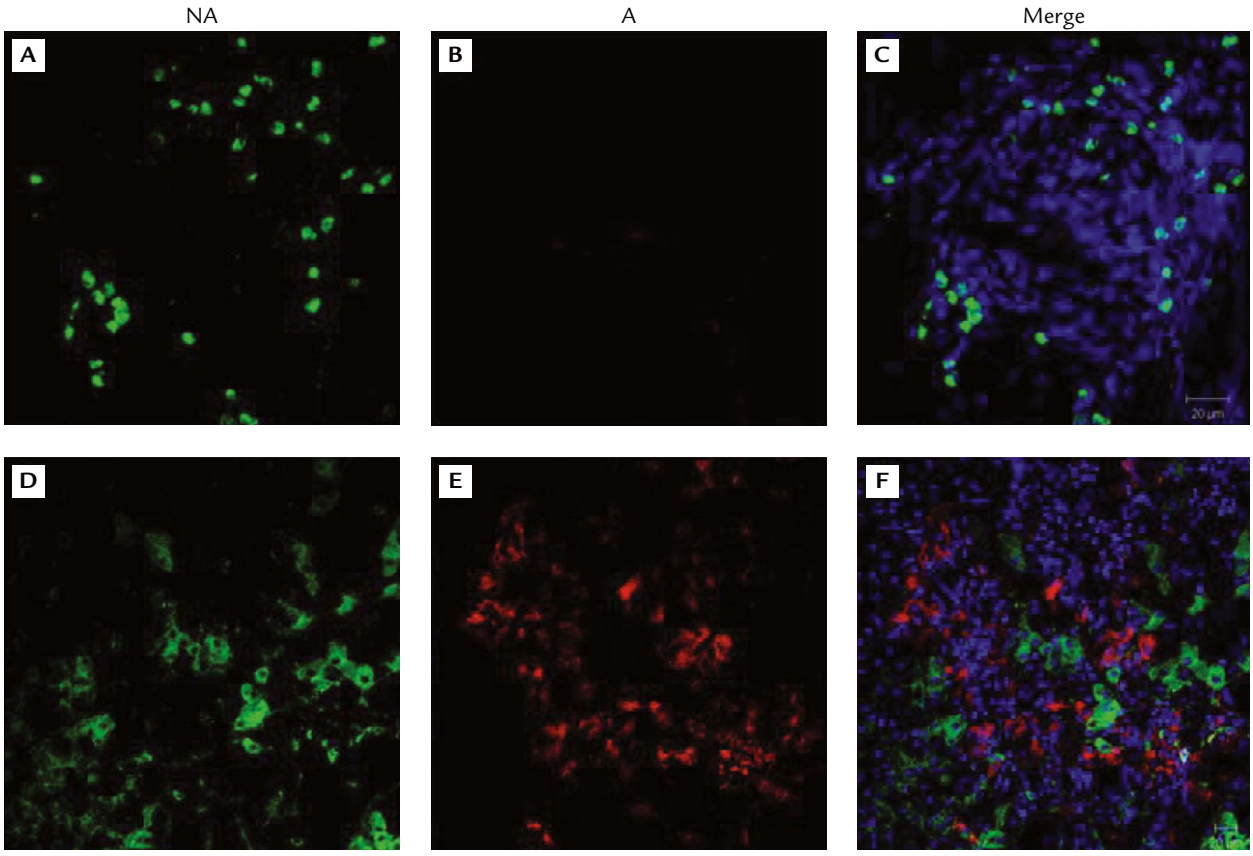

Figure 3 Immunofluorescence staining for detection of catecholamine-storing cells in the acupoint area of CV12 (A–C) and the adrenal gland (D–F). The left panel shows noradrenaline (green). In the center panel is adrenaline (red) and the right panel shows the merged images where cell nuclei (blue) were stained with DAPI. (C) Only noradrenaline-storing cells were found inside a globular tissue formation. Magnification 500×. (D–F) Noradrenaline- and adrenaline-storing chromaffin cells in the adrenal medulla. Magnification 250×.

Table 1 Morphological characteristics of catecholamine-storing cells in CV12 acupoints

Sample no	Cell distribution	Cell size (μm)	Cell shape	Presence of catecholamines
1	Cluster	15–20	Oval	NA
2	Single	10	Round	NA
3	Single	15	Round	NA
4	Cluster	10–15	Oval	NA
5	Single	15–20	Oval	NA
6	Cluster	10–15	Oval	NA

NA = noradrenaline.

4. Discussion

Using double immunofluorescence staining, we have found NA-storing cells in the dermal layer of rabbit skin which were located nearby the CV12 acupoint. NA-storing cells were randomly scattered as single cells as well as existing in small clusters in a globular tissue formation surrounded by blood vessels and capillaries. To the best of our knowledge, the major sources of catecholamines are the adrenal medulla and postganglionic fibers of the sympathetic nervous system. However, several studies showed that epidermal keratinocytes and melanocytes [26–28], activated macrophages, lymphocytes and bone marrow-derived mast cells [29–31] also have the capability to synthesize and store catecholamines such as dopamine, NA and A. If we classify these cells by their contents, then all of them belong to

mixed cell types, whereas in adult adrenal glands there are two different chromaffin cell types storing NA or A [32,33]. In our study we observed only NA-storing cells located in the dermal layer of rabbit skin after double immunostaining with polyclonal antibodies against NA and A (Figures 3A, B, C). Thus the cells observed in our study are different from the epidermal and immune cells in their catecholamine content.

According to the acupuncture map for animals an acupoint should be located precisely on the locus defined by proportional measurement based on anatomical landmarks [24]. However, in clinical situations, the acupuncturist typically searches for the acupoint around the standard text acupoint location, implicitly assuming that the acupoint location is different in each person and changes in time [34]. We therefore used a relatively large skin site (1 cm^2) for the CV12 acupoint which was sectioned in the z-plane for determination of the locations of NA-storing cells as specific markers for acupoints. Our findings showed that the positions of NA-storing cells were distributed about 2–4 mm away from the CV12 acupoint, but not exactly under the anatomically-inferred point. These data can be interpreted in different ways. On one hand, different locations of NA-storing cells in the acupoint site of the skin indicate that each animal has individual positions for acupoints. Apparently, the acupoints have similar locations in the skin but are not the same in different animals. For example, even when the anatomical distribution of blood vessels or peripheral nerves in the skin of two different people are compared, their distributions will not be identical. Thus our findings show that acupuncture charts, especially for animals, merely indicate approximate positions of acupuncture points.

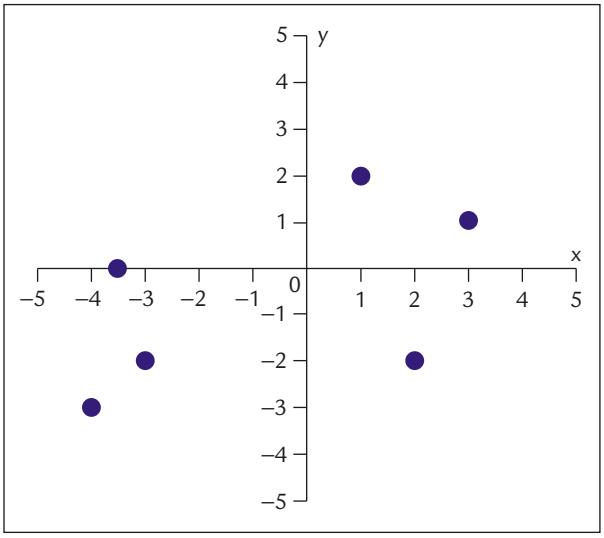

Figure 4 The locations of noradrenaline (NA)-storing cells in six representative samples are presented in two-dimensional plane. A distance from the center to the points of the location of NA-storing cells is given in mm.

On the other hand, based on the morphometric data on the distribution of globular tissue formation with NA-storing cells located near acupoints, we think this structure can be activated by mechanical stimulation of acupuncture needles. According to the biomechanical model suggested by Langevin, any manipulation of acupuncture needles can result in a deformation of connective tissue inducing various changes in cellular and tissue levels [35]. Once the needle has become mechanically coupled to the tissue, subsequent needle manipulation (rotation or pistoning) may pull on collagen and elastin fibers, resulting in deformation of the extracellular connective tissue matrix. This matrix deformation may be transduced into local cells present within connective tissue, with a wide variety of downstream effects ranging from cell contraction, gene expression, and secretion of paracrine or autocrine factors to neuromodulation of afferent sensory input [36].

Thus biomechanical stimulation may be mediated by connective tissue fibers which surround the globular tissue formation in rabbit skin. Winding of collagen and elastin fibers in response to needle rotation may induce a release of NA by catecholamine-storing cells, nitric oxide (NO) in endothelial cells or nerve fibers exposed to mechanical forces etc. Indeed, recent studies have demonstrated that skin NA concentration and ^{3}H-NA release in acupoints of BL56,

Table 2 The locations of noradrenaline-storing cells in the CV12 acupoint area (1 cm^2) of rabbits

Sample no	Position (x, y), mm	Depth (μm)	Diameter (μm)	Location in the skin
1	x=−3, 5, y=0	500–600	200	Dermis
2	x=2, y=−2	300–400	170	Dermis
3	x=−3, y=−2	400–500	190	Dermis
4	x=−4, y=−3	400–500	150	Dermis
5	x=1, y=2	500–600	170	Dermis
6	x=3, y=1	500–600	160	Dermis

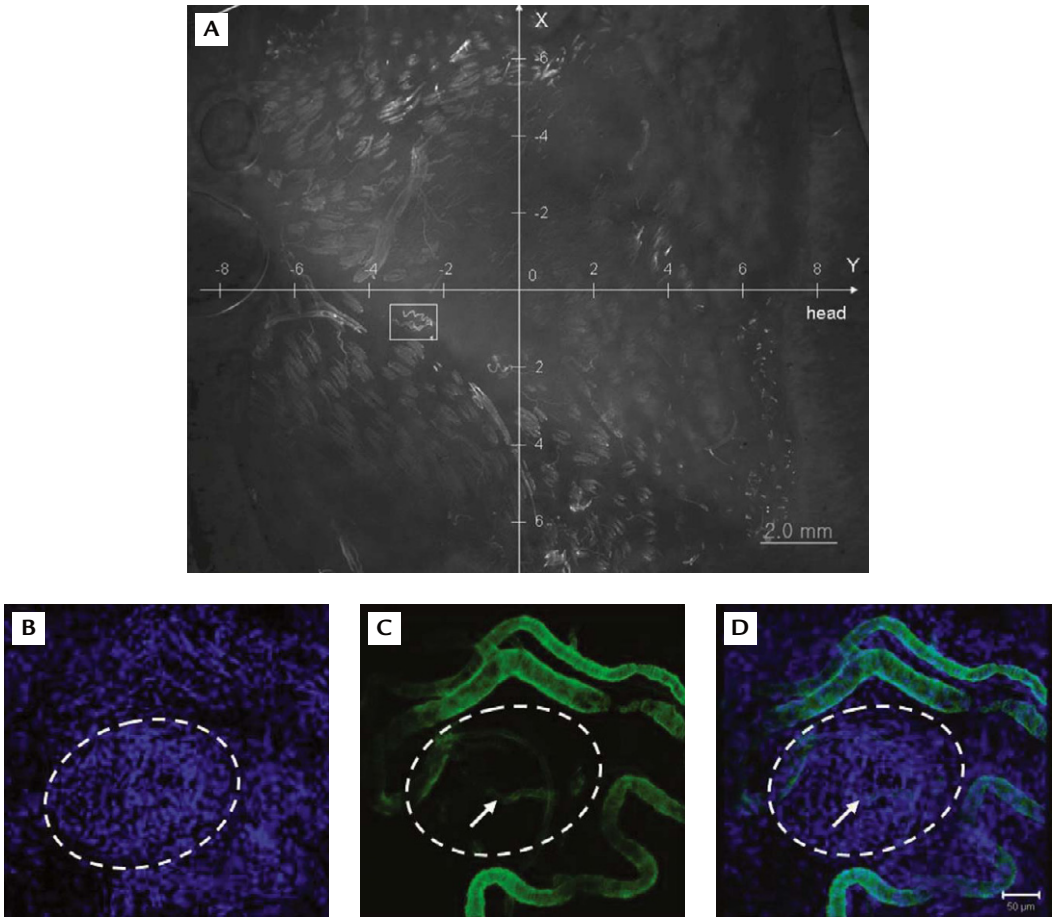

Figure 5 (A) Stereomicroscopic image of a skin section of CV12 clearly showing the location of a globular tissue formation surrounded by blood vessels and capillaries (rectangle). The position of the globular structure in the section was defined by X- and Y-direction, in the point of intersection x=0.9 mm and y=−2.6 mm. (B–D) Magnified view of the rectangle in Figure 5A. (B) Fluorescence image showing a distribution of cell nuclei (blue) in the globular formation (circle). (C) Blood vessels and capillaries (arrow). (D) Merge image of Figures B and C showing the globular formation (circle) surrounded by blood vessels. Small blood capillaries are observed in the central part of the globular formation. Magnification 250×.

PC6 and GV6 were higher than those in non-acupoints and non-meridian control points. It was shown that significant release of NA by peripheral nerves is mediated by enhanced NO level in the acupoints [21]. Chen and his associates used quantitative biochemical methods for measurement of NA in whole skin, implying sympathetic peripheral nerve fibers as the main source of NA. However, it was shown that the levels of catecholamines per unit weight were significantly higher in epidermis than in the dermis and whole skin [37]. This means that epidermal cells of the skin can contribute significantly to the concentration of NA in biochemical tests. Thus the use of analytical methods for measurement of hormones in whole skin can reflect catecholamine levels. In our study, we used qualitative immunochemical techniques for *in situ* localization of catecholamine-storing cells in rabbit skin. Specific globular tissue formation with NA-storing cells was located in the dermal layer of rabbit skin. Apparently, NA-storing cells in the skin may represent an additional source of catecholamines, in particular NA, which may be released during mechanical stimulation with an acupuncture needle.

Thus our findings on the localization of NA-storing cells in acupoints is in line with the trend of discoveries of additional catecholamine sources, and also confirm the essential characteristics of the Bonghan corpuscles that are the proposed anatomical component of acupoints [38]. These results could lead to the scientific elucidation of the neurophysiological effects of acupuncture treatments by the coupling of the nervous system through neurotransmitter hormones to needling stimulation.

Acknowledgments

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