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RESEARCH ARTICLE

Tracing Mercox Injected at Acupuncture Points Under the Protocol of Partial Body Macerations in Mice

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Abstract
We used for the first time a vascular casting material to take advantage of a simple tracing procedure and to isolate the peculiar features of acupuncture point injections. The polymer Mercox was injected into the skin of a dead mouse at acupuncture points along the bladder meridian lines. After a partial maceration of the whole body with a potassium-hydroperoxide solution, we anatomized it under a stereomicroscope to trace the injected Mercox. Many organs were checked to determine whether or not they contained some Mercox tracing. Connections between the injection sites along the acupuncture points were observed. Two to three layers of Mercox in a plate shape were found

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tracing method

under the skin at the acupuncture points, and Mercox travelled throughout the adipose tissue, the fascia, and the parietal and visceral serous membranes inside the organ's parenchyma. The casting material Mercox used with a modified partial maceration procedure is a promising method for visualizing the routes of the meridian system and the primo vascular system. The routes for Mercox are different from those of the blood and lymphatic vessels.

1. Introduction

The meridian system, which is a basic framework for acupuncture treatment, has not yet been clearly defined physiologically or anatomically in the human and animal body. Many investigations into the system have been done to build mechanisms and tracings for acupuncture and meridians. In the 1960s, Kim [1] studied the meridian system and suggested physical and anatomical substrates for the meridians and the acupuncture points. He aimed to elucidate the question of the circulation of some liquid through the meridians. At that time, he used radioactive tracers to identify meridians and adopted electrophysiological methods for his research to gain access to the excitability and conductivity of the meridian system. Recently, his works were revived, and the primo vascular system (PVS) was suggested as an extension of the acupuncture meridian [2].

Tracing the meridians with radioisotopes has been attractive in attempts to visualize the network of the entire system. Radioactive pathways of migration of hypodermically injected technetium-99m were investigated around the points of low electrical resistance [3]. The detected radioactive pathways were found not to be the result of diffusion of a radiotracer through nerves, veins, or lymphatic vessels, but to coincide with the acupuncture meridians. The pathways of the acupuncture meridians in the human body were investigated through the injection of radioactive tracers at acupuncture points [4], and the pathways were found to be distinct from either the lymphatic or blood vascular routes. Under the hypothesis that the acupuncture points are physically connected to the internal organs, contrast agents for magnetic resonance imaging, such as gadolinium and fluorine, have been used as tracers for the meridians [5].

Regarding the flow along the meridians, low-hydraulic-resistance channels were investigated in experiments using pig models and were compared with nonmeridian areas [6]. The fluid mechanics model was suggested to explain tissue fluid flow in the limb connective tissue of the human body and as a possible mechanism for acupuncture signal transmission along meridians [7]. Many studies have shown the existence of hypodermic migration channels, independent of lymphatic and blood vessels, for radiotracers along the meridians. However, the tracing of radioactive isotopes floating under the skin has not been clear enough to identify the anatomic structures in the body [8].

One of the most commonly used tissue-processing techniques for vascular imaging is casting of the vasculature by intravascular injection of a filling agent, followed by corrosion of the surrounding tissue for imaging by stereoscopic microscopy and/or scanning electron microscopy [9]. Vascular corrosion casting has been used as a powerful method for visualizing the morphologies of vasculature structures such as the blood vessels of the cerebral cortex [10], the initial lymphatics in human skin biopsy specimens [11], and the vascular architecture of the mouse embryo [12]. The casting method using Mercox has provided quantitative and morphological information on the microvasculature of organs and tissues [13] and for the visualization and the stereological assessment of blood and lymphatic vessels [14]. The method has also been used to study morphological changes induced in the hepatic microvasculature by ischemia-reperfusion injury [15], and to classify tumor vessels in a mammary carcinoma [16]. In more recent works, the method has been used for *in vivo* studies with powerful imaging technologies such as magnetic resonance angiography, complementing their limited spatial resolutions [17].

In this study, we designed for the first time completely new types of experiments for meridian studies with modified corrosion casting, as well as what we already published for other organs [18]. For first time we used dead animals during tracing of meridians' pathways as well as a completely different tracing material, Mercox. Mercox, as a casting material, was injected at the acupuncture points of mice skin. In order to trace the polymerized Mercox in the body, a partial maceration was done with a potassium hydroperoxide solution. Our working hypothesis was that Mercox, as one of the best vascular casting materials, would fill the vessels of the skin. Our thinking about the behavior of the Mercox was a result of the very good vascularization of the skin and the use of dead animals for this experiment. In this case, when the animals are already dead, the routine paths of distributing of the outgoing signals should not be working as well as in live animals. Surprisingly, the patterns of Mercox permeability under the deep skin and their directionality were observed along the meridians, and morphological comparisons between injections at acupuncture points and those at non-acupuncture points were made.

2. Materials and methods

2.1. Animals

Twelve female Institute of Cancer Research (ICR) mice aged 8—10 weeks were purchased from Dooyeol-Biotech, Inc. (Seoul, Korea) and were divided into experimental and control groups. Procedures involving the animals and their care conformed to the institutional guidelines and were in full compliance with current policies. The experiments were carried out within 1 or 2 days after the purchase of the mice. Two mice were injected with Mercox at only one

acupuncture point. For the sacrifice of the mice, an overdose of urethane was injected intraperitoneally.

2.2. Preparations of Mercox and maceration

Red and blue Mercox (acrylic polymer in primary form; Ladd Research Industries, Williston, VT, USA) were obtained with a catalyst for polymerization. Mercox (0.5 mL) of each color and the catalyst (0.02 mg) were mixed just before the injections to each mouse. Disposable syringes of 1-mL volume with disposable needles (30G $\times$ 1/2''; Sungshin Medical Co., Bucheon-Si, Korea) were used for injections to each mouse. The mixed solution with a single color of Mercox (0.1 mL) was injected at each specified point (4 points in total) on the skin subcutaneously. The injections were made after confirming the cessation of heart activity of the mice. All the injections to an individual mouse were completed within 2 minutes. The Mercox-injected mouse was kept for 30 minutes before being put into a solution for maceration. Potassium hydroperoxide (KOH, 3%, Duksan Pure Chemicals Co., Ansan-si, Korea) was dissolved in distilled water to macerate the mouse's body or organ samples. In the case of the whole mouse, partial maceration was done by placing the mouse in the potassium-hydroperoxide solution for 36 hours.

2.3. Acupuncture points

For the positioning of animal acupuncture points on the surface of the skin, we adopted the transpositional method, which corresponds to the anatomic sites of human acupuncture points. Although there is still no generally accepted agreement on the specific locations of animal acupuncture points among researchers, the positioning method was chosen because the anatomical differences between humans and animals had been considered [19,20]. The injected acupoints are as follows: BL15, BL20, BL25, and BL28; they are on the two bladder meridian lines, one on the left and the other on the right of the dorsal part of the body. The bladder meridian lines reside symmetrically on both sides approximately 3 mm laterally from the midline along the spine near the fifth thoracic vertebra, the 12^{th} thoracic vertebra (the 11^{th} thoracic vertebra in humans), the fourth lumber vertebra, and the second sacrum, as shown in Fig. 1. Blue and red Mercox were injected in a zigzag series at the acupuncture points. The zigzag injecting series means, at first, injecting a series of blue Mercox at some acupoints, and then another series of red Mercox between the blue ones as shown in Fig. 1. Nonacupuncture points on both sides, approximately 10 mm laterally from the midline, were also chosen as the control points and are marked in Fig. 1.

2.4. Procedure and observations

The procedures for the experiments were as follows: after the mice had been sacrificed, hair on the dorsal part of the mouse's skin was removed using a shaver. The positions of the acupoints were determined and marked by palpations of the body. First, blue Mercox was prepared for injection at four points; second, red Mercox was prepared for injection at another four acupuncture points. We injected each color of Mercox in the same pattern for the control experiments. All the injections were completed within 2 minutes. After waiting for 30 minutes, we put the whole body of the mouse in a glass container of 3% potassium-hydroperoxide solution for 36 hours for partial macerations. For observations under the skin and Mercox tracing, a razor blade and small scissors were used for layer-by-layer cutting into deep parts of the body. After the body had been completely traced, we removed the organs for more maceration and separate observations. After a full search on the dorsal part of the whole body had been completed, we removed organs such as the heart, kidney, adrenal gland, liver, lung, pancreas, spleen, and spinal cord. All the organs were macerated for 24 hours in separate Petri dishes with 3% potassium-hydroperoxide solution in order to see the distribution of the Mercox inside the organs.

The instruments used for microscopic observations were a stereomicroscope (SZX12; Olympus, Tokyo, Japan), an optical microscope (BX51; Olympus, Tokyo, Japan), and a polarizing microscope (KSM-BA3; Samwon, Goyang-si, Korea).

3. Results

After the 36-hour partial maceration had been completed, the skin of the dorsal part of the mouse was cut and peeled off from the tail to the head using a razor and a scissors. The dorsal view of a partially-macerated mouse with Mercox injections at the acupoints is shown in Fig. 2A. A control mouse that had undergone the same procedure except for the injections is shown in Fig. 2B. In the case of acupuncture-point injection, long tracks along the spine from the neck to the upper part of tail were observed. The tracks were more clearly exposed when we made a full maceration with the whole body of the mouse (Fig. 2C). One advantage of the partial maceration in this experiment was that it made the observations possible while keeping the positions of polymerized Mercox inside the tissue. Otherwise, the mouse would have completely melted away so that the positions of the Mercox would have been lost.

At each injection site, due to isotropic diffusion, a mass of Mercox was polymerized as a primary object. The mass of Mercox had a plate shape with a size of $>$ 3 mm in diameter and 1 mm in thickness. One of the typical differences between an acupuncture point and a control point was the number of layers of Mercox under the deep skin tissue. The first layer was in the dermal part of the skin, and the second one, in the hypodermal part. As for the acupoints, Mercox had at least two more layers under the primary object. In some cases, a third layer, superficial fascia covering the muscles, was found. The layers were tightly connected, however, they could be separated layer by layer. As shown in Fig. 3A, layers of blue and red Mercox can be seen on both the left and the right sides at the acupuncture point BL15. There was some directionality, protruding Mercox, for the connections between the injection points under the deepest layers, as shown in Fig. 3B. Closer observations for the surface of the polymerized Mercox inside the body showed numerous clusters

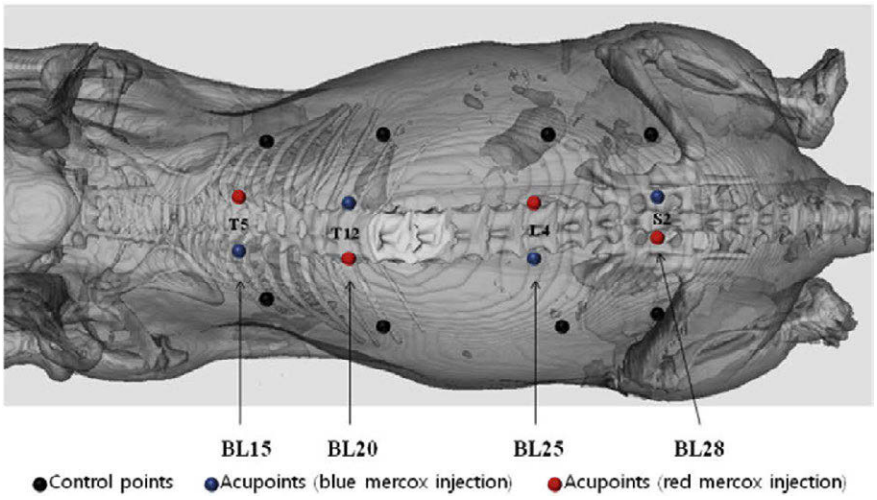

Figure 1 Positions of acupoints on the mouse for injection of Mercox.

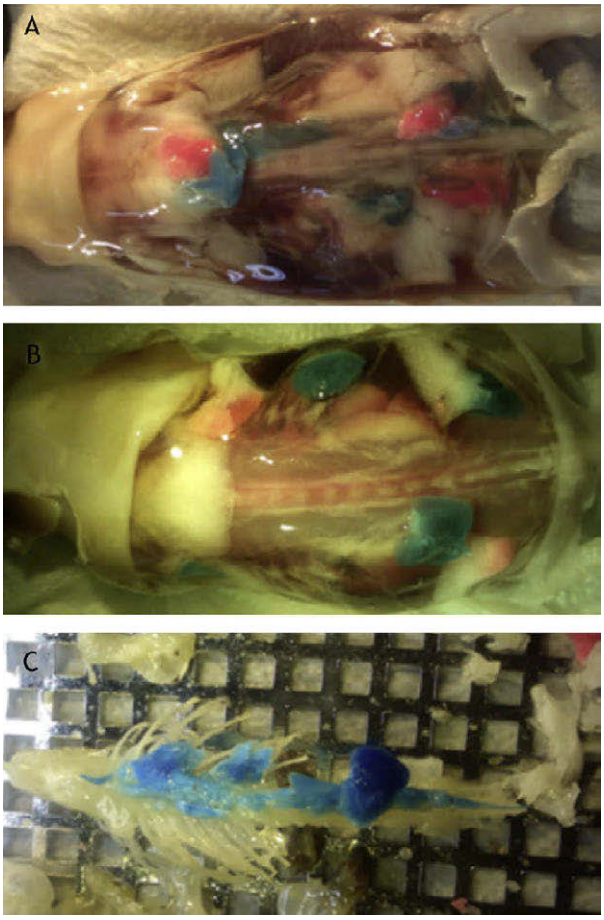

Figure 2 Dorsal views of the mouse after partial macerations and peeling off of the skin. (A) Acupuncture point injections. (B) Control point injections. (C) The mouse after full macerations with acupuncture point injections.

of adipocyte cells, as shown in Figs. 3C and 3D, for blue and red Mercox, respectively.

In spite of the short time interval for the polymerization (within 10 minutes), we found that a large amount of Mercox had already reached into many internal organs. Some structures maintained their vascular or cellular shapes, however, the Mercox found in internal organs had mostly fragmented into many pieces. Precise tracing of Mercox was difficult, but the routes were mainly through adipose tissue, then to the loose connective tissue between the muscles, and the parietal and visceral serous membranes, as well as peritoneum and pleura, and, very surprisingly, the polymer reached the parenchyma of the organs. We found no tracers inside the organ's parenchyma, however, we have established the existence of small colored particles from Mercox into the parenchyma of all investigated organs. Fig. 4 presents stereomicroscopic images of some of the organs containing Mercox.

One of the smallest polymerized Mercox particles inside the organs was found in the spleen, as shown in Fig. 5A. It had a very thin plate shape with a protrusion of 10 μm in size. By cutting the spine, we also found Mercox inside the vertebra and the spinal cord covers, as shown in Fig. 5B. Serial sections along the vertebra showed some long tracks inside the spinal vertebra. The experiments with one acupuncture point injection showed a very short Mercox line consisting of fat cells, which continued after the injecting point, but reached neither the deep structures of the skin nor the loose connective tissue, fascia, and organ parenchyma.

4. Discussion

We found some tendency to have Mercox connections between the acupuncture points along the meridians under the deep skin. At a cellular level, those connections were made by protrusions of Mercox-clustered adipocyte cells. That the adipocytes could absorb Mercox was totally unexpected, but an amazing finding. We confirmed that one of the adipocytes with a 4,6-diamidino-2-phenylindole (DAPI)-stained nucleus was filled with Mercox. One of the reasons that the adipocytes are filled with Mercox is probably due to dissolution of the polymer by lipids. More details on this subject are presented in another paper where we showed that the Mercox can penetrate into some

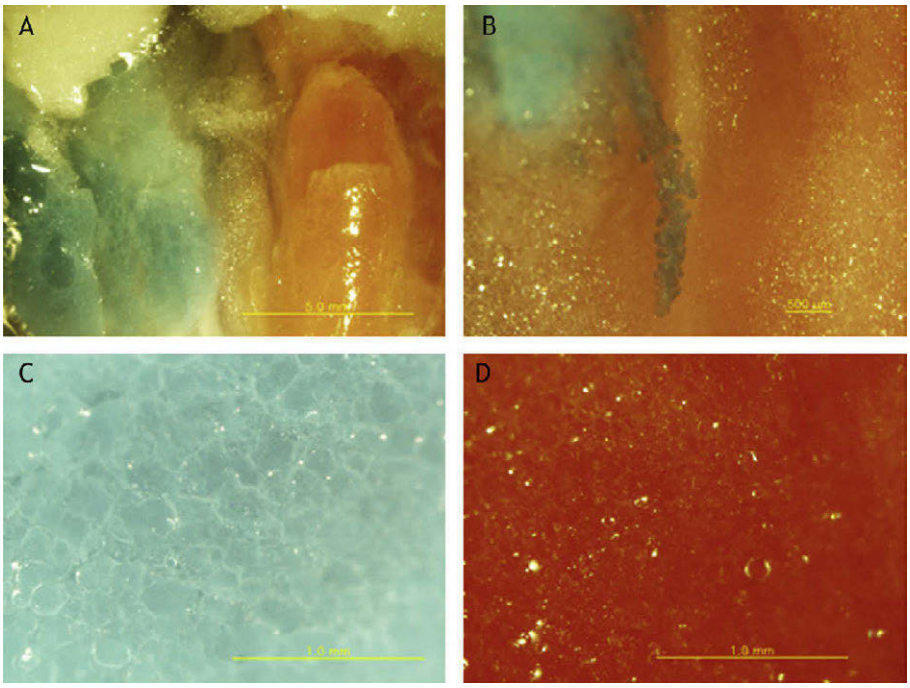

Figure 3 Mercox injected under the skin. (A) Layers of blue and red Mercox on the left and right at BL15, respectively. (B) The Mercox protrusion along the meridian direction. Surfaces of (C) blue and (D) red Mercox. Clusters of adipocytes can be seen.

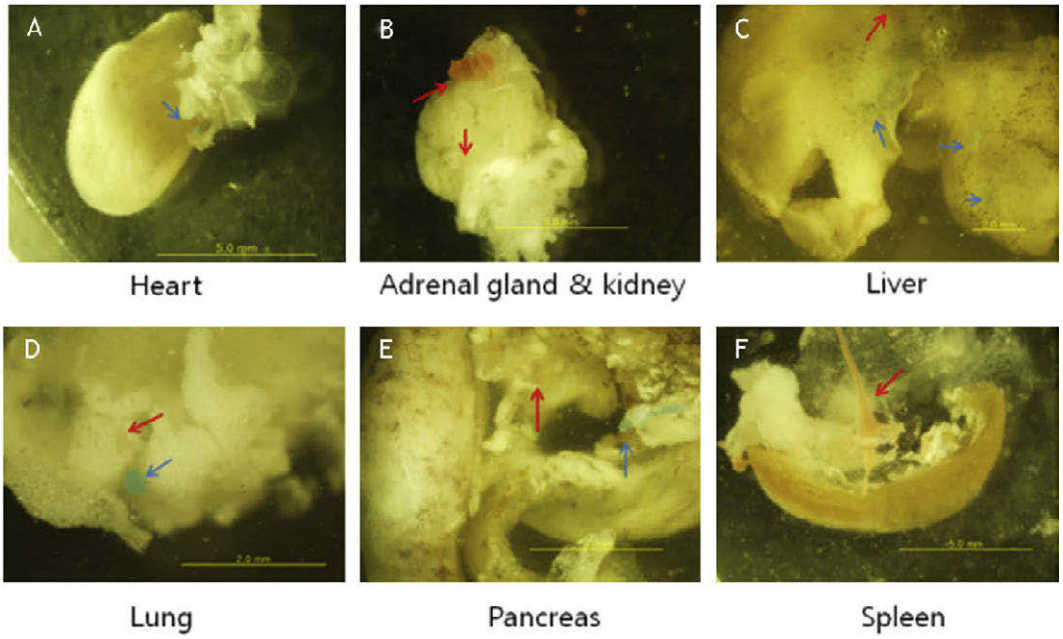

Figure 4 Red and blue Mercox found in various organs. (A) Heart. (B) Adrenal gland and kidney. (C) Liver. (D) Lung. (E) Pancreas. (F) Spleen.

cell membranes and are able to fill different anatomical structures [18]. Because the PVS is embedded most frequently in the fat tissue, and because the serous membranes and loose connective tissue are normal anatomical structures where the PVS course through, as the PVS is followed the vessels and the nerves and is abundant in the loose connective tissue, fat tissue, serous membranes, and fascia reaching every single cell [21, 22] we could suggested that the routes of Mercox throughout the skin injection follow not only the meridians but also the PVS distribution. The findings could be indirect evidence that PVS and meridians have unbreakable bonds. Another reason why Mercox has a very special distribution into the skin, quite different from the distribution of the skin, blood, and lymph vessels, could be low hydraulic resistance channels along meridians described by Zhang et al. [23,24]. The

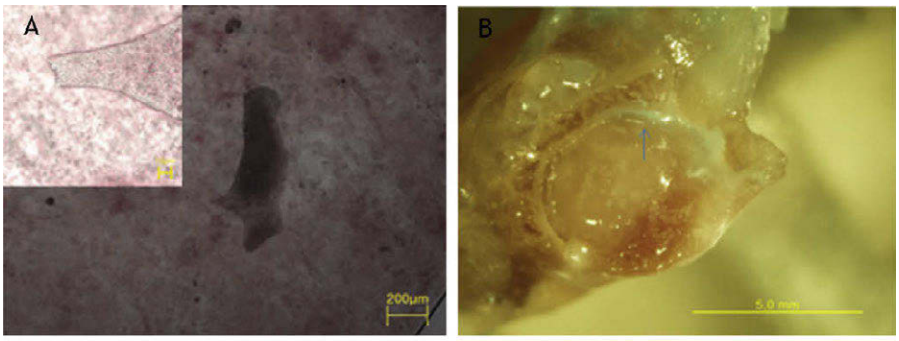

Figure 5 Mercox. (A) Red Mercox found inside the spleen. The inset shows a magnified view of the part of the Mercox. (B) Blue Mercox found inside a spinal vertebra.

speed at which Mercox reaches the organ's parenchyma is outstanding, despite nonfunction of any other systems. Therefore, special conditions and structures may permit the penetration of the tracer from skin to the organs' parenchyma for a short time immediately after the animal's death. Probably, the characteristics of the polymer Mercox permit its distribution to the meridians because of low hydraulic resistance. Another distinguishing feature of an acupuncture point is that the injected Mercox forms several layers under the skin. This feature, which is induced by the properties of Mercox, may reflect some anatomical difference under the skin between the acupuncture points and the control points. At the control points, Mercox was distributed only in the dermal layer.

Mercox injection into the skin under the acupuncture points in dead animals is a new approach in the search for meridian trace pathways and the first attempt in the world. To date, only casting injections to predefined vascular routes and cavities have been done. In the current experiments, the size of most diffused Mercox was $>$ 3 mm in diameter at the injection site, which is large enough to cover other possible acupuncture points in nearby surrounding areas. A smaller quantity of Mercox injection would help a new type of vascular structure, such as the PVS [2], to be visualized under the skin under more controlled conditions.

Regarding tracing an intermediate pathway from the skin to the internal organs, our procedure was suitable for distinguishing the route of the polymer and was able to confirm the final products of the migrations. Precisely tracing possible pathways may be possible using computed tomography and scanning electron microscopy. With radiopaque Mercox, the time course of Mercox migration can be visualized and traced in the body under *in vivo* conditions. Microinjections of Mercox, combined with a scanning electron microscopic search, can be used so that finer structures related to acupuncture and meridians, such as the PVS, can be visualized in future experiments.

The traces that we found in this study confirmed the hypothesis made by Bai et al. [25] concerning fasciology. The main topic of this hypothesis is that fascia, including fat tissue and loose connective tissue, is the anatomic base of the meridian system. Mercox may be able to pass through adipose and loose connective tissue, fascia, and visceral and parietal serous membranes to reach the parenchyma of the organs, making traces as it does so.

In conclusion, a vascular casting material with modified partial maceration procedures is a promising method for visualizing the routes and the tracers of the meridian system. The route of Mercox is different from that of the blood and the lymphatic pathways.

Disclosure statement

The authors declare that they have no conflicts of interest and no financial interests related to the material of this manuscript.

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